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*It is characteristic of Science and Progress that they continually open new fields to our visions.*—Pasteur.

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# QUANTITATIVE DETERMINATIONS OF SOME OF THE BIOCHEMICAL CHANGES PRODUCED BY A SAPROPHYTIC ANAEROBE[1]

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## INTRODUCTION

The differentiation and classification of microbial species is certainly the most difficult of all problems in bacteriology. When one finds what he considers a new species he is at once aware of the lack of means to characterize it adequately so that it may be recognized by others. At the same time the similarities that it presents to other types, which are certainly distinct, may lead to confusion, when an attempt is made to place it properly among species already described. This confusion is more marked in the case of the anaerobic than of the aerobic types. This is probably due to the fact that anaerobic types are supposed to be very unstable in their characteristics and less care has been used in describing them; and also to the fact that many mixed cultures of anaerobes have been described as pure cultures.

Probably some of this confusion is due to the fact that too much emphasis has been placed on one factor, such as the form of the colony, or the location and size of the spore. It is a well known fact that colony formation is quite variable, in many cases being completely altered by the consistency of the medium. Again the location of the spore varies in many of the individuals of the same culture.

Bacteriological experience has taught us not to rely too much upon one factor, but rather to classify microörganisms according

[1] Contribution no. 41 from the Bacteriological Laboratories of the Kansas Agricultural Experiment Station.

to a group of factors. The fermentation of both carbohydrates and proteins should be considered, and a certain amount of cultural and morphological variation should be allowed for each group.

## DESCRIPTION OF THE ORGANISM USED IN THE PRESENT STUDY

In the study of anaerobic bacteria isolated from spoiled canned asparagus we have found one type which predominated and which could be distinguished by colony formation in the deep agar column. This organism was a Gram positive, spore-bearing, anaerobic rod; motile, by means of 12 to 15 peritrichic flagella. The size of the majority upon plain agar was 0.80 to 0.90 by 2.50 to 3.50 microns. They were somewhat longer and more slender upon glucose media.

The surface colonies are very thin and transparent at first, but gradually assume a more opaque granular center from which the surface growth extends over the medium for several millimeters. The edge is quite regular in outline, slightly raised from the medium. The colonies later become more irregular with root-like projections extending from the edge of the colony while similar projections extend down into the medium below the colony. These colonies usually show a central nucleus which at first is more opaque than the remainder of the colony. The colonies are transparent but in most cases there is a whitish appearance in older cultures. Under low magnification the surface colonies appear to be finely granular but remain nearly colorless.

The deep colonies are spherical at first and more opaque than those on the surface. They later develop root-like outgrowths or may become quite woolly in some instances, especially if the medium is soft. Older colonies tend to become somewhat less opaque as they increase in size. Some of the colonies are somewhat lenticular in outline and have woolly or root-like outgrowths from one side only. This appearance is described as "*En grenade*" by Weinberg and Seguin (1918). Some of the cultures of this group appear ragged in outline, not having distinct projections but with a very irregular surface.

The organism would be considered to be proteolytic in its action upon protein-containing media. Gelatin is liquefied rapidly and completely. Inspissated serum is rapidly cleared, followed by nearly complete digestion. In alkaline meat media there is a rapid growth with the development of much gas and a distinctly unpleasant but not putrefactive odor. There is slight digestion of the meat at the surface. This, however, was not marked in any of the cultures. The surface layers of the meat are darkened, while the lower layers retain their original color. There is a heavy, whitish growth of bacteria on the surface of the meat in very old cultures. About 1 or 2 mm. below the surface is a blackened zone. Traces of hydrogen sulphide are found in meat cultures. The liquid remains neutral or slightly acid and the particles of fat on the surface appear to be partially disintegrated. Growth in Von Hibler's brain media is very similar.

Growth in milk is rapid. There is very little or no gas formed. The casein is thrown down in a soft clot which is usually completely digested within four days. With some of the cultures, however, digestion is never complete. The whey separates and in a short time becomes of a clear light amber color, or yellowish and turbid, according to the culture. The reaction is neutral or slightly acid on standing, and the fat layer becomes disintegrated, probably due to saponification. The cultures have a strong cheesy to butyric acid odor. Some of the cultures are somewhat more putrid and upon long standing, especially if enclosed in a jar, there is a slight odor of ethyl-butyric or of valerianic acid. Cultures seem to differ considerably in this respect with age.

In the egg medium, made according to the formula given by Robertson (1916), the organism grows slowly. The first indication of growth is the appearance of blackening on the bottom of the tube. This is followed in some cases by the formation of a soft, yellowish clot which becomes fissured and may settle out, leaving a clear, yellowish, transparent fluid. Some of the cultures do not cause coagulation even on long standing, and those cultures which form a clot, fail to produce further change, except in a few cases, in which there is a marked shrinking of the clot. If the medium was made slightly acid nearly all the cultures caused

the formation of the clot, with darkening at the bottom of the tube; but aside from the shrinking no other change occurred.

Indol, phenol, skatol or alcohol were not found. There was a slight reduction of nitrates to ammonia. The pH as determined colorimetrically in Clark and Lubs media ranged from 5.8 to 6.2. Cultures were all negative for the methyl-red and the Voges-Proskauer reactions.

Considerable difficulty was encountered in determining which of the carbohydrates were actually fermented. The work was first done by the use of Durham's fermentation tubes, using meat infusion broth made sugar free by fermentation with *B. saccharolyte*. This medium was placed in tubes and autoclaved. To it was added 1 per cent of the carbohydrate to be examined and the tubes were incubated for the detection of contamination. Just before inoculation they were heated and cooled rapidly in running water. They were then inoculated with a large loopful of a four day potato-peptone culture of the organism. The cultures were placed over phosphorous in an anaerobic jar and incubated for three days at 37°C.

Difficulty was sometimes encountered, however, in determining the fermentation of such carbohydrates as lactose, inulin, etc., due to the fact that the closed arm of the tube would contain a bubble of gas from one to three millimeters in diameter. The reaction of the medium in these cases was but little changed, being sometimes made slightly acid. In other cultures, however, distinct amounts of acid were formed. Hiss's serum water plus these carbohydrates gave similar results.

Table 1 shows the essential features of these tests. The "+" indicates a distinct amount of acid or gas. If a bubble of gas is present it is marked with a "b."

From our preliminary tests it was decided that we were dealing with organisms belonging to the *B. sporogenes* group (Metchnikoff, 1908). There were differences between the various cultures, but these were not marked. Some cultures always produced a clouding of the medium, others grew mostly on the bottom of the tube. Certain cultures produced more blackening of the egg medium than others. Generally speaking all the cultures exhibited the same characteristics.

TABLE 1

| ORGANISMS | FORM | GRAM STAIN | MOTILITY | LOCATION OF SPORES | LIQUEFICATION OF GELATIN | LIQUEFICATION OF SERUM | DIGESTION OF CASEIN | BLACKENING OF MEAT | BLACKENING OF BRAIN | GLUCOSE | | LEVULOSE | | GALACTOSE | | SUCROSE | | LACTOSE | | MALTOSE | | GLYCEROL | | MANNITOL | | DULCITOL | | SALICIN | | STARCH | | ARABINOSE | | XYLOSE | | $H_2S$ | PHENOL | SKATOL | AMMONIA | ACETIC ACID | BUTYRIC ACID | VALERIC ACID | ALCOHOL | $CO_2$ | $H_2$ | $CH_4$ | TYPE OF COLONY |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | a | R | a | R | a | R | a | R | a | R | a | R | a | R | a | R | a | R | a | R | a | R | a | R | | | | | | | | | | | | |
| 2 B | Rod | + | + | s. t. | + | + | + | tr. | tr. | + | + | + | + | + | + | − | b | − | b | + | + | + | + | tr. | + | − | b | + | + | + | + | − | b | − | b | − | − | − | + | + | + | ? | − | + | + | − | Irregular wooly |
| 5 B | Rod | + | + | s. t. | + | + | + | tr. | tr. | + | + | + | + | + | + | − | b | − | b | + | + | + | + | tr. | + | − | b | + | + | + | + | − | − | − | b | − | − | − | + | + | + | ? | − | + | + | − | Woolly |
| 28 B | Rod | + | + | s. t. | + | + | + | tr. | tr. | + | + | + | + | + | + | − | b | − | b | + | + | + | + | tr. | + | − | b | + | + | + | + | − | − | − | − | tr. | − | − | + | + | + | − | − | + | + | − | Woolly |
| B. spor. I | Rod | + | + | s. t. | + | + | + | tr. | tr. | + | + | + | + | + | + | − | b | tr. | − | + | + | ? | + | − | + | − | − | + | + | + | + | − | − | − | − | − | − | − | + | + | + | | − | + | + | − | Woolly |
| B. spor. II | Rod | + | + | s. t. | + | + | + | tr. | tr. | + | + | ? | + | + | + | − | b | − | b | + | + | tr. | + | tr. | + | − | 2% | tr. | + | + | + | − | − | − | − | − | − | − | + | | | | | + | + | − | Irregular wooly |
| B. spor. III | Rod | + | + | s. t. | + | + | + | + | + | + | + | ? | + | + | + | − | 3% | − | b | + | + | + | + | − | b | − | − | + | b | − | b | − | b | − | − | − | − | − | + | | | | | | | | Irregular not wooly |
| B. spor. Henry (4) | Rod | + | + | t | + | | + | + | | + | + | + | + | − | − | − | − | − | − | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − | | | | | | | | | | | Woolly |

"s. t." and "t" under heading "location of spores" refers to subterminal and terminal.

All the cultures were completely non-toxic when grown on brain and liver broth and on brain—saline for different lengths of time and injected subcutaneously into guinea-pigs in 1 cc. amounts. Table 2 shows the results of one series.

TABLE 2

*Results of animal inoculation*

| CULTURE | AGE OF CULTURE | DAYS TO DEATH OF ANIMAL |
|---|---|---|
| | *days* | |
| 2 B | 6 | Found dead on 30th day (colon bacilli from heart blood) |
| 5 B | 16 | Alive after 6 weeks |
| 28 B | 23 | Alive after 6 weeks |
| 37 B | 34 | Alive after 5 weeks |

TABLE 3

*Agglutination reaction*

| RABBIT IMMUNIZED AGAINST CULTURE | CULTURE | DILUTION OF IMMUNE SERUM | | | | | | | | NORMAL SERUM | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1:25 | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 | 1:1600 | 1:3200 | 1:50 | 1:200 |
| 2 B | 2 B | *10 | 10 | 10 | 10 | 10 | 10 | 5 | 0 | 2 | 0 |
| 5 B | 5 B | 8 | 10 | 10 | 10 | 10 | 10 | 8 | 2 | 0 | 0 |
| 28 B | 28 B | 10 | 10 | 10 | 10 | 10 | 10 | 8 | 0 | 0 | 0 |
| 2 B | 5 B | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 0 | 4 | 0 |
| 2 B | 28 B | 10 | 10 | 10 | 10 | 10 | 5 | 0 | 0 | 2 | 0 |
| 5 B | 2 B | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 3 | 3 | 0 |
| 5 B | 28 B | 10 | 10 | 10 | 10 | 10 | 8 | 0 | 0 | 2 | 0 |
| 28 B | 2 B | 10 | 10 | 10 | 10 | 10 | 10 | 3 | 0 | 0 | 0 |
| 28 B | 5 B | 10 | 10 | 10 | 10 | 10 | 10 | 2 | 0 | 1 | 0 |
| 5 B | B. spor. I | 10 | 10 | 10 | 10 | 10 | 9 | 3 | 0 | 3 | 0 |
| 5 B | B. spor. II | 8 | 10 | 10 | 10 | 10 | 8 | 0 | 0 | 2 | 0 |
| 5 B | B. bot. II | 10 | 10 | 10 | 5 | 0 | 0 | 0 | 0 | 2 | 0 |

* Figures show amount of agglutination. 10 = complete.

Several of the cultures of this organism were also serologically similar to known cultures of *B. sporogenes*, as shown by table 3. Their non-toxic nature and the appearance of colonies in deep agar would differentiate them from *B. botulinus*, to which species they are otherwise very similar.

## QUANTITATIVE ANALYSIS OF BIOCHEMICAL CHANGES

Since the qualitative results left us in doubt concerning the fermentation ability of these organisms, we attempted to determine these points by quantitative tests.

Wolf and Harris (1917a; 1917b; 1918) and Wolf and Telfer (1917) and Wolf (1918; 1919) have conducted exhaustive experiments upon the biochemistry of pathogenic anaerobes from war wounds, using quantitative methods. These authors did not attempt to determine the ability of these organisms to ferment many of the carbohydrates ordinarily used for the separation of microörganisms into groups, and studied only those found in wound infection. They did, however, collect some very valuable information concerning some of the activities of a large number of the more common pathogenic anaerobes.

We hoped, by tests of this sort, to determine some more accurate and rapid method for the classification of anaerobes in general, and those in particular with which we were working. Since the dangers of botulism from the eating of canned foods is more prevalent than formerly, it is quite essential that bacteriologists should obtain all the information possible concerning the groups of spore-bearing anaerobic bacteria which may be found in food. As has been mentioned above, the classification of anaerobes is very much confused at the present time; and since their fermentative ability, as determined by the ordinary qualitative methods is difficult to determine accurately, the only apparent method of obtaining real differences is by quantitative methods.

We have, therefore, attempted to utilize the following quantitative indications of difference: (1) Amount and kind of gas produced from various carbohydrates; (2) amount and kind of acid produced from various carbohydrates; (3) amount of proteolytic action as determined by formation of ammonia and amino-acids.

The first condition to be fulfilled is to obtain a medium which will be as constant as possible in composition, and one in which organisms of this type are able to grow. The second condition

is to obtain an apparatus by means of which the various changes may be noted and recorded over a series of days, since the fermentations change with the age of the culture to a marked extent. We hoped to obtain more accurate information by this method although the amount of work necessary is multiplied in proportion to the number of determinations made.

Of course, it is impossible under all conditions to make the medium absolutely the same. However, we have attempted to do this as far as possible, by obtaining enough material in one lot for an entire series of tests. The same asparagus was used throughout; and for the carbohydrate fermentation tests, we used a 2 per cent peptone-water to which, after sterilization, was added 1 per cent of the carbohydrate in question, except in case of glucose of which 0.5 per cent was used. We used peptone-water rather than broth because this could be made more uniformly, and did not require an adjustment of the reaction. Unfortunately these organisms would not grow in any of the mineral solutions usually recommended for the culture of nitrogen fixing anaerobes, so their action in a medium of known composition could not be determined.

In their experimental work Wolf and Harris used a large 2-liter bottle for culturing the anaerobes. This bottle was fitted with a two-holed rubber stopper through which passed two glass tubes, one of the tubes extending into the space above the liquid and one into the liquid itself and nearly to the bottom of the bottle. The container was exhausted and the culture allowed to develop in vacuum until the space above the liquid was filled with gas. Samples were removed at intervals by means of the tubes. The presence of the gases was utilized to force out enough liquid for an analysis.

The apparatus described by these investigators could not be used by us, since our cultures did not grow very rapidly, and we invariably found that a leak had occurred and air had entered. Also, we experienced difficulty with contamination, in sampling according to their method. Aside from the difficulties mentioned, the apparatus recommended by Wolf and his associates would probably give more uniform results for a single series of examina-

tions than the one used in this work, since but one culture container was used and all the samples taken from it. Results could not be exactly duplicated, however, by the use of this apparatus.

All our cultures were placed in a vacuum as complete as we could obtain. Great difficulty was experienced in avoiding leaks, and in obtaining a culture vessel which would withstand sterilization and still be strong enough not to be crushed under the high external pressure. At the time this work was done, special glassware could not be purchased on the market and "pure" gum rubber tubing that would hold a vacuum was not obtainable. Numerous samples of so-called pure gum rubber, were secured, but none of them would hold a high vacuum for twenty-four hours, although they were coated with celloidin and various cements recommended for this purpose. We desired enough culture material for several tests and this required a large culture vessel.

## DESCRIPTION OF THE APPARATUS

The following apparatus (fig. 1) was finally devised and met these difficulties very well.

A quart milk bottle (N) was used as a culture vessel. To this was added from 300 to 500 cc. of the culture medium and the bottle was then autoclaved at 20 pounds pressure for thirty minutes. The asparagus was merely suspended in tap water, 100 grams in 300 cc. of water. Fresh whole mixed milk was used. The potato medium was made by adding 100 grams of potato to 300 cc. of water. The potato was carefully selected, washed, peeled and passed through a meat grinder; and the potato pulp was washed in running water for several hours. For the carbohydrate fermentation tests a 2 per cent peptone solution was used. Three hundred cubic centimeters of this solution were placed in the bottles and sterilized. The carbohydrates were sterilized in 10 per cent solution and added at the time of inoculation.

As an inoculum, we used 1 cc. of a four day old potato-peptone-water culture of the organism grown at 37°C. The four day culture seemed to give the best results. At first smaller amounts

were used but in many cases we failed to obtain growth. Care was always used not to add bits of potato.

Just before inoculation, the bottles of media were heated in the steamer and then cooled as rapidly as possible to drive out the air. Care was always exercised not to shake up the medium during the inoculation and sealing process. After inoculation, the cotton stoppers were removed and the bottles were plugged with a No. 8, two-holed rubber stopper, fitted with two glass tubes. One of these tubes was constricted near the upper end and served as an attachment for the vacuum pump, and for sealing after the container had been exhausted. The second tube was bent to serve as a manometer tube (P) as indicated in figure 1. The rubber topper was fitted with the tubes, wrapped in gauze and then covered with a thick layer of cotton. The lower end of the manometer tube and the upper end of the straight tube were plugged with cotton and all autoclaved at twenty pounds pressure for an hour. It is necessary to wrap the cotton stopper in the mouth of the bottle, and the rubber stopper with gauze to prevent bits of cotton adhering to them, since this would prevent a perfect seal.

After inoculation, the cotton stopper was discarded, and the rubber stopper carrying the tubes, was unwrapped and well flamed with a Bunsen flame. The rubber stopper was then pushed firmly into the neck of the bottle and covered with a thick layer of rubber cement. Thin blocks of wood were placed on the top of the stopper, and all wired in place with No. 16 iron wire.

After the bottle had been fitted, it was placed in a special incubator at 37°C. and immersed in a copper tank (M) containing heavy mineral oil. The lower end of the manometer tube was placed in a dish containing mercury, after removal of the cotton plug. The bottle was then exhausted as much as possible with a Geryk pump. When the mercury column ceased to rise in the manometer tube the tube attached to the pump was sealed at the constriction with a blast lamp. By this method we could obtain practically a complete vacuum as determined by the temperature and barometric pressure at the time. Figure 1 shows the details of this apparatus.

A series of cultures was prepared in exactly the same way and an analysis made at intervals as indicated in table 4.

## METHODS OF ANALYSIS

*Gas analysis.* (*Total volume.*) The amount of gas could be calculated from the manometer readings and reduced to normal temperature and pressure by using the following formula:

$$V = \frac{273\,V(P - T)}{760\,(273 + t)}$$

in which $V$ = volume, $P$ = barometric pressure in mm. Hg., $T$ = vapor tension of water,[2] 760 = normal pressure, 273 = absolute temperature.

Determinations of the total volume of gas were recorded upon the bottle which remained in the incubator for the longest time. Manometer readings were taken on all the cultures at each interval but not all of these are included in the tables, since they would give no additional information as to the course of the fermentation.

*Method for collection and analysis of gas.* To obtain samples of gas, uncontaminated with air, the mercury pump was attached to the slender sealed tip (D) shown in figure 1. A tight fitting rubber stopper was first placed over the sealed tip and the rubber connection forced carefully over the tip until the metal tube came well down over the tip. This was wired firmly in place. Next the large rubber tube, which served as a receptacle for mercury, was drawn down and fitted over the stopper and wired in place. This large tube was filled with mercury to cover the connections completely.

The mercury pump was filled with mercury as completely as possible and the tube leading from the mercury pump to the connection with the culture container was exhausted with the Geryk pump. When the entire apparatus was exhausted, the connec-

[2] The vapor tension of water was used in all cases. This probably varied to some extent as the fermentation proceeded and new volatile products were formed. From the fact, however, that these were of unknown value, it was possible to use only the vapor tension of water in our calculations.

tion at D was given a slight bend to break the sealed tip. (If the tip has not been sealed too close to the lower part of the constriction it is very easily broken.) When the tip is broken there is an immediate fall of the mercury in the pump and a corresponding rise of the mercury column in the manometer tube. The gas is pumped out of the culture bottle and collected in a container over the mercury pump. The pumping is continued until the mercury column in the manometer tube becomes stationary.

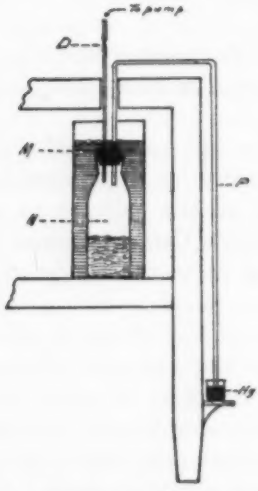


FIG. 1. SHOWING ARRANGEMENT OF CULTURES IN THE INCUBATOR

FIG. 2. SHOWING DETAILS OF THE CONNECTION BETWEEN THE CULTURE BOTTLE AND THE MERCURY PUMP

This is usually very close to the theoretical vacuum, and required about fifteen or twenty minutes. By this means it was possible to obtain the dissolved gases as well as those which had collected above the liquid. The liquid boiled vigorously at 37°C. and the water vapor probably aided in washing the gases out of the bottle.

After the gas had all been forced into the collecting bottle the connection between this bottle and the gas analysis apparatus

was opened and the mercury in the gas burette lowered. By this means a sample of gas was drawn in, measured and analysed. Figure 2 shows the details of the connection made at (D) figure 1.

The efficiency of our apparatus and pump was judged by the amount of oxygen in the gas obtained for analysis. In a few analyses we obtained a slight reduction in volume after absorbing the gas with alkaline pyrogallate solution. In such a case we calculated the air in the gas by multiplying the amount of decrease by five and making corrections in the calculation. These amounts were, however, usually nearly within the error in reading our burette. The apparatus used for the analysis of the gas is described by Burrell and Siebert (1913) and is a slight modification of that described by Haldane.

*Calculations.* The following example will show the calculations necessary in this connection. These were used throughout and for that reason the figures for each analysis are not included.

21.65 cc. of gas taken
5.63 cc. after $CO_2$ absorption
16.02 cc. of $CO_2$
5.63 cc. after $O_2$ absorption
0.00 cc. of $O_2$
18.45 cc. of $CO_2$ free air added
24.08 cc. total volume
16.85 cc. after combustion
7.23 cc. decrease due to combustion
7.19 cc. after $CO_2$ absorption
0.04 cc. of $CO_2$ due to combustion
4.82 cc. of hydrogen ($\frac{2}{3}$ of decrease)
73.99 per cent $CO_2$
22.26 per cent $H_2$
3.71 per cent residual gas (by measurement)

The 0.04 cc. of $CO_2$ due to combustion was disregarded, since this amount is insignificant.

After the completion of the gas analysis, air was allowed to enter the apparatus through a cotton plug. The bottle was then removed from the incubator and the examination completed. The bottle was opened as carefully as possible and a sample taken for cultural purposes. From this sample stains, shakes and plates were made. The cultures were incubated several days at

37°C. From the shakes we could detect the presence of anaerobes, and plates showed the presence of aerobes. After we began the use of the present apparatus no contamination has been noted and we were always able to culture the anaerobe if growth had occurred.

Samples were also taken for the determination of ammonia, amino-acids and volatile fatty acids.

*Ammonia determinations.* The ammonia determinations were made by distilling in the presence of magnesium oxide. At first we tried the aeration method but even after eight hours we were able to obtain tests for ammonia with Nessler's reagent. After the gas analysis was made there was insufficient time for the aeration. It has been found in this laboratory, in working with fermenting mixtures of soil, etc., that there is a close correlation between duplicate samples run by the aeration and distillation methods. With small amounts of ammonia, the distillation methods, usually gave somewhat higher results than the aeration method; while with large amounts of ammonia present the results were reversed. When small amounts of ammonia (10 to 20 mgm. per 100 grams soil) were present the distillation method gave 3.6 per cent more ammonia than the aeration method. When large amounts of ammonia (60 to 75 mgm. per 100 grams soil) were present the aeration method gave 4.0 per cent more ammonia then the distillation method.

*Amino-acid determination.* Amino-acids were determined by the Van Slyke method. These determinations were complicated by the presence of very large amounts of ammonia. Van Slyke (1911) recommends the following method for the removal of ammonia: The ammonia can be removed by distillation with $Ca(OH)_2$ under diminished pressure, using a 10 per cent suspension until a slight excess is present, as shown by turbidity and alkaline reaction of the solution. The apparatus is evacuated to 30 mm. or less of mercury, and distillation continued for one-half hour at 45 to 50°C. Alcohol is added to prevent foaming.

We were unable to use this method because of lack of time and the difficulty of removing all the ammonia from the medium, As large amounts of ammonia were present and the temperature

of the laboratory was high (usually 25 to 35°C.), a correction for ammonia was necessary.

The following method was used in making these corrections. A 2 per cent peptone solution containing known amounts of standard ammonium hydroxide was placed in the Van Slyke apparatus and the amount of nitrogen determined in the usual manner. The difference between the peptone solution alone and a peptone solution plus ammonia was considered to be due entirely to ammonia.

Van Slyke states that about 15 per cent of the ammonia nitrogen present will be determined as amino-acid nitrogen at 20°C. in fifteen minutes. We obtained the following percentages by averaging the results obtained from duplicate determinations made on different dates.

Per cent of ammonia nitrogen that would be recorded as amino-acid nitrogen at different temperatures for the time used (five minutes).

At 21°C. = 34.2 per cent
At 23°C. = 39.6 per cent
At 25°C. = 44.9 per cent
At 27°C. = 50.6 per cent
At 29°C. = 55.7 per cent
At 31°C. = 61.1 per cent
At 33°C. = 66.3 per cent
At 35°C. = 71.6 per cent
At 37°C. = 77.0 per cent

This means that the above per cents of the total ammonia nitrogen present would be recorded as amino-acid nitrogen at the various temperatures recorded.

It will be noted that these figures are considerably higher than those recorded by Van Slyke. The results obtained give a fairly uniform curve and were obtained under our experimental conditions. Of course, they were obtained by the use of a comparatively simple mixture, while in actual tests we were dealing with an extremely complex mixture. However, all the fermentation mixtures were different and it would have been impossible to simulate them closely.

TABLE 4

*Showing results of chemical examination*

| PRODUCTS FORMED | ACTION ON ASPARAGUS (100 GRAMS IN 300 CC. WATER) | | | | ACTION ON ALKALINE EGG MEDIUM | | | |
|---|---|---|---|---|---|---|---|---|
| | Days incubated | | | | Days incubated | | | |
| | 2 | 4 | 6 | 8 | 2 | 5 | 8 | 12 |
| Total cubic centimeters of gas | 148.0 | 251.0 | 257.0 | 280.0 | 66.0 | 77.0 | 102.0 | 112.0 |
| Daily cubic centimeters of gas | 74.0 | 51.0 | 3.0 | 11.0 | 33.0 | 4.0 | 8.0 | 2.5 |
| Per cent of $CO_2$ | 98.6 | 97.2 | 97.1 | 95.3 | 64.9 | 71.3 | 70.1 | 73.7 |
| Per cent of $H_2$ | 0 | 0 | 0.5 | 3.3 | 26.4 | 17.8 | 23.9 | 16.8 |
| Per cent residual gas | 1.6 | 1.9 | 1.8 | 1.4 | 8.7 | 10.8 | 6.0 | 9.5 |
| Cubic centimeters of $CO_2$ in gas | 146.0 | 244.0 | 250.0 | 267.0 | 43.0 | 55.0 | 72.0 | 83.0 |
| Daily $CO_2$ in gas | 73.0 | 49.0 | 3.0 | 9.0 | 22.0 | 4.0 | 6.0 | 3.0 |
| Cubic centimeters of $H_2$ in gas | 0 | 0 | 1.0 | 9.0 | 17.0 | 14.0 | 24.0 | 19.0 |
| Daily $H_2$ in gas | 0 | 0 | 0.5 | 4.0 | 9 | −1.0 | 3.0 | −1.0 |
| Ratio $H_2/CO_2$ | — | — | 1/250 | 1/29.0 | 1/2.4 | 1/4.0 | 1/3.0 | 1/4.4 |
| Ammonia-N milligrams per 100 cc. | 19.0 | 28.0 | 33.6 | 34.4 | 17.3 | 54.6 | 65.4 | 74.4 |
| Daily ammonia-N milligrams per 100 cc. | 7.0 | 4.5 | 2.8 | 0.4 | 5.5 | 12.4 | 3.6 | 2.2 |
| Amino-acid-N milligrams per 100 cc. | 27.83 | 35.14 | 61.86 | 69.20 | 67.56 | 103.35 | 93.08 | 87.75 |
| Daily amino-acid-N milligrams per 100 cc. | 3.04 | 3.65 | 13.36 | 3.67 | 26.85 | 11.93 | −3.42 | −1.33 |
| Cubic centimeters N/20 NaOH to neutralize volatile acids per 100 cc. | 9.10 | 11.70 | 11.00 | 8.20 | — | 6.80 | 9.60 | 15.60 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily | 3.05 | 1.30 | −0.35 | −1.40 | — | 2.26 | 0.93 | 1.50 |
| Per cent of volatile acids in fractions of distillate: 10 cc. | 6.5 | 5.1 | 5.4 | 7.3 | — | 6.30 | 8.8 | 11.5 |
| 20 cc. | 13.1 | 10.2 | 10.8 | 13.4 | — | 10.5 | 14.7 | 20.5 |
| 30 cc. | 19.7 | 15.4 | 15.3 | 19.5 | — | 14.6 | 19.6 | 26.9 |
| 40 cc. | 26.1 | 22.5 | 21.6 | 27.4 | — | 17.7 | 24.6 | 33.3 |
| 50 cc. | 32.5 | 27.9 | 27.0 | 32.3 | — | 21.7 | 28.1 | 38.4 |
| 100 cc. | 48.3 | 44.4 | 45.1 | 51.2 | — | 39.6 | 48.5 | 52.5 |
| 200 cc. | 68.1 | 66.6 | 65.1 | 73.1 | — | 60.4 | 62.0 | 65.3 |
| 300 cc. | 79.0 | 78.6 | 78.2 | 85.2 | — | 72.5 | 76.7 | 71.8 |
| 400 cc. | 85.6 | 85.4 | 79.6 | 90.1 | — | 80.8 | 85.6 | 76.9 |
| 500 cc. | 89.9 | 90.5 | 81.4 | 92.5 | — | 87.6 | 88.5 | 80.7 |
| | Before inoculation: Ammonia-N 5.0 mgm. per 100 cc. Amino-acid-N 20.75 mgm. per 100 cc. Volatile acids 3.0 cc. N/20 per 100 cc. | | | | Before inoculation: Ammonia-N 6.2 mgm. per 100 cc. Amino-acid-N 13.85 mgm. per 100 cc. Volatile acids 0.0 cc. N/20 per 100 cc. | | | |

TABLE 4—*Continued*

| PRODUCTS FORMED | ACTION ON WHOLE MILK MEDIUM | | | ACTION ON 2 PER CENT PEPTONE WATER | | | | |
|---|---|---|---|---|---|---|---|---|
| | Days incubated | | | Days incubated | | | | |
| | 5 | 8 | 12 | 3 | 5 | | 11 | 14 |
| Total cubic centimeters of gas | 132.0 | 194.0 | 241.0 | 43.0 | 61.0 | 77.0 | 102.0 | 110.0 |
| Daily cubic centimeters of gas | 26.0 | 21.0 | 12.0 | 14.0 | 9.0 | 5.0 | 8.0 | 3.0 |
| Per cent of $CO_2$ | 76.8 | 78.2 | — | 72.7 | 76.5 | 81.7 | 86.2 | 86.1 |
| Per cent of $H_2$ | 15.0 | 17.5 | — | 17.3 | 15.2 | 12.6 | 5.2 | 8.9 |
| Per cent of residual gas | 8.2 | 4.3 | — | 10.0 | 8.3 | 5.7 | 8.6 | 5.0 |
| Cubic centimeters of $CO_2$ in gas | 101.0 | 152.0 | — | 31.0 | 47.0 | 63.0 | 88.0 | 95.0 |
| Daily $CO_2$ in gas | 20.0 | 17.0 | — | 10.0 | 8.0 | 5.0 | 8.0 | 2.0 |
| Cubic centimeters of $H_2$ in gas | 20.0 | 34.0 | — | 7.0 | 9.0 | 10.0 | 5.0 | 10.0 |
| Daily $H_2$ in gas | 4.0 | 5.0 | — | 2.0 | 1.0 | 0.3 | −1.6 | 1.6 |
| Ratio $H_2/CO_2$ | 1/5.1 | 1/4.5 | — | 1/4.4 | 1/5.3 | 1/6.3 | 1/17.6 | 1/5.9 |
| Ammonia-N milligrams per 100cc, | 72.6 | 77.4 | 83.6 | 98.0 | 101.0 | 104.0 | 108.6 | 111.5 |
| Daily ammonia-N milligrams per 100 cc | 12.7 | 1.6 | 1.5 | 32.0 | 1.3 | 1.0 | 1.3 | 0.9 |
| Amino-acid-N milligrams per 100 cc | 83.14 | 92.45 | 124.47 | 132.98 | 112.04 | 103.74 | 33.48 | 32.56 |
| Daily amino-acid-N milligrams per 100 cc | 11.80 | 3.11 | 7.99 | 19.59 | −10.47 | −2.77 | −23.42 | −0.64 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc | 39.60 | 40.90 | 44.60 | 10.95 | 12.60 | 15.80 | 15.35 | 13.57 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily | 6.91 | 0.43 | 0.92 | 3.20 | 0.55 | 1.06 | −0.15 | −0.59 |
| Per cent of volatile acids in fractions of distillate: 10 cc. | 8.5 | 8.9 | 8.9 | 7.20 | 6.6 | 5.8 | 6.3 | 7.2 |
| 20 cc. | 15.6 | 16.2 | 15.2 | 12.4 | 11.9 | 11.2 | 11.8 | 13.6 |
| 30 cc. | 21.7 | 22.6 | 21.9 | 19.1 | 16.7 | 16.3 | 16.5 | 19.6 |
| 40 cc. | 27.3 | 27.4 | 27.9 | 24.3 | 21.5 | 20.9 | 21.3 | 23.8 |
| 50 cc. | 32.8 | 31.4 | 32.8 | 29.6 | 26.3 | 25.6 | 25.6 | 27.9 |
| 100 cc. | 49.5 | 48.0 | 47.1 | 42.3 | 41.4 | 40.3 | 40.6 | 42.8 |
| 200 cc. | 66.1 | 65.6 | 61.0 | 60.4 | 58.2 | 58.1 | 59.5 | 61.1 |
| 300 cc. | 74.2 | 74.9 | 67.8 | 72.2 | 69.7 | 68.2 | 69.4 | 71.2 |
| 400 cc. | 79.3 | 80.2 | 73.6 | 81.4 | 77.7 | 75.9 | 76.5 | 79.2 |
| 500 cc. | 82.6 | 84.6 | 77.6 | 84.6 | 83.4 | 81.8 | 81.6 | 84.8 |
| | Before inoculation: Ammonia-N 9.0 mgm. per 100 cc. Amino-acid-N 24.14 mgm. per 100 cc. Volatile acids 5.05 cc. N/20 per 100 cc. | | | Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 74.20 mgm. per 100 cc. Volatile acids 1.35 cc. N/20 per 100 cc. | | | | |

TABLE 4—*Continued*

| PRODUCTS FORMED | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 0.5 PER CENT GLUCOSE | | | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT LACTOSE | | |
|---|---|---|---|---|---|---|
| | Days incubated | | | Days incubated | | |
| | 3 | 6 | 10 | 2 | 6 | 9 |
| Total cubic centimeters of gas | 208.0 | 312.0 | 333.0 | 82.0 | 128.0 | 133.0 |
| Daily cubic centimeters of gas | 69.0 | 34.0 | 5.0 | 41.0 | 12.0 | 2.0 |
| Per cent of $CO_2$ | 62.2 | 68.4 | 90.0 | 75.9 | 84.5 | 88.2 |
| Per cent of $H_2$ | 33.7 | 27.3 | — | 16.9 | 8.8 | 8.6 |
| Per cent residual gas | 4.1 | 4.3 | — | 7.2 | 6.7 | 3.2 |
| Cubic centimeters of $CO_2$ in gas | 129.0 | 213.0 | 300.0 | 62.0 | 108.0 | 117.0 |
| Daily $CO_2$ in gas | 43.0 | 28.0 | 22.0 | 31.0 | 15.0 | 3.0 |
| Cubic centimeters of $H_2$ in gas | 70.0 | 85.0 | — | 14.0 | 11.0 | 11.0 |
| Daily $H_2$ in gas | 23.0 | 5.0 | — | 7.0 | −1.0 | 0 |
| Ratio $H_2/CO_2$ | 1/1.8 | 1/2.5 | — | 1/4.5 | 1/9.8 | 1/10.6 |
| Ammonia-N milligrams per 100 cc | 22.6 | 55.0 | 78.5 | 76.0 | 97.0 | 100.0 |
| Daily ammonia-N milligrams per 100 cc | 6.8 | 10.8 | 5.9 | 37.1 | 5.2 | 1.0 |
| Amino-acid-N milligrams per 100 cc | 79.81 | 97.95 | 113.12 | 109.76 | 116.14 | 65.40 |
| Daily amino-acid-N milligrams per 100 cc | 7.57 | 6.04 | 3.79 | 21.68 | 1.59 | −16.91 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc | 24.2 | 32.5 | 59.8 | 12.75 | 11.85 | 10.30 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily | 7.6 | 2.8 | 6.8 | 6.10 | −0.25 | −0.52 |
| Per cent of volatile acids in fractions of distillate: 10 cc. | 2.5 | 4.9 | 6.0 | 5.3 | 6.8 | 5.9 |
| 20 cc. | 5.0 | 9.0 | 11.4 | 10.1 | 12.6 | 11.2 |
| 30 cc. | 6.7 | 14.8 | 16.4 | 14.9 | 17.9 | 16.2 |
| 40 cc. | 8.4 | 19.7 | 20.4 | 19.8 | 22.9 | 20.8 |
| 50 cc. | 10.3 | 23.8 | 24.1 | 23.7 | 27.6 | 24.7 |
| 100 cc. | 22.6 | 27.9 | 35.4 | 39.5 | 41.4 | 39.6 |
| 200 cc. | 42.7 | 41.9 | 54.5 | 58.4 | 62.5 | 51.1 |
| 300 cc. | 56.1 | 60.0 | 66.8 | 69.4 | 74.0 | 62.9 |
| 400 cc. | 62.8 | 72.4 | 75.6 | 76.0 | 81.6 | 69.9 |
| 500 cc. | 67.7 | 80.6 | 81.2 | 80.8 | 86.9 | 75.0 |
| | Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 57.1 mgm. per 100 cc. Volatile acids 1.4 cc. N/20 per 100 cc. | | | Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 66.39 mgm. per 100 cc. Volatile acids 0.55 per 100 cc. | | |

TABLE 4—*Continued*

| PRODUCTS FORMED | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT SUCROSE | | | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT SALICIN | | |
|---|---|---|---|---|---|---|
| | Days incubated | | | Days incubated | | |
| | 4 | 7 | 9 | 4 | 7 | 9 |
| Total cubic centimeters of gas | 5.0 | 28.0 | 42.0 | 43.0 | 208.0 | 228.0 |
| Daily cubic centimeters of gas | 1.0 | 8.0 | 7.0 | 11.0 | 55.0 | 10.0 |
| Per cent of $CO_2$ | — | 18.3 | 52.4 | — | 61.9 | 65.8 |
| Per cent of $H_2$ | — | 70.9 | 35.4 | — | 34.1 | 30.7 |
| Per cent of residual gas | — | 10.8 | 12.2 | — | 4.0 | 3.5 |
| Cubic centimeters of $CO_2$ in gas | — | 5.0 | 22.0 | — | 129.0 | 150.0 |
| Daily $CO_2$ in gas | — | 1.6 | 8.5 | — | 4.3 | 16.0 |
| Cubic centimeters of $H_2$ in gas | — | 19.9 | 14.9 | — | 71.0 | 70.0 |
| Daily $H_2$ in gas | — | 6.6 | −2.5 | — | 24.0 | −0.3 |
| Ratio $H_2/CO_2$ | — | 4.0/1 | 1/1.5 | — | 1/1.8 | 1/2.1 |
| Ammonia-N milligrams per 100 cc. | 4.0 | 51.5 | 99.5 | 21.0 | 45.0 | 81.5 |
| Daily ammonia-N milligrams per 100 cc. | 0.5 | 15.8 | 24.0 | 4.8 | 8.0 | 18.3 |
| Amino-acid-N milligrams per 100 cc. | 71.81 | 77.46 | 83.79 | 61.81 | 77.46 | 83.79 |
| Daily amino-acid-N milligrams per 100 cc. | −0.30 | 1.88 | 3.16 | 1.18 | 5.21 | 3.16 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. | 3.00 | 17.30 | 13.40 | 5.90 | 20.20 | 42.60 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily | 0.61 | 4.77 | −1.95 | 1.34 | 4.77 | 11.20 |
| Per cent of volatile acids in fractions of distillate: 10 cc. | 5.0 | 6.9 | 6.8 | 1.7 | 5.5 | 5.8 |
| 20 cc. | 9.8 | 12.1 | 12.8 | 3.4 | 9.5 | 11.5 |
| 30 cc. | 13.3 | 16.8 | 18.4 | 5.1 | 13.5 | 16.4 |
| 40 cc. | 15.0 | 20.8 | 23.1 | 6.8 | 16.9 | 20.8 |
| 50 cc. | 16.7 | 24.2 | 27.3 | 7.6 | 19.4 | 24.8 |
| 100 cc. | 29.9 | 39.8 | 44.9 | 12.7 | 33.3 | 40.2 |
| 200 cc. | 46.6 | 57.7 | 63.7 | 19.5 | 56.6 | 59.3 |
| 300 cc. | 56.6 | 68.1 | 77.3 | 25.5 | 62.5 | 70.8 |
| 400 cc. | 64.9 | 75.0 | 87.1 | 28.9 | 69.9 | 77.4 |
| 500 cc. | 71.6 | 80.0 | 94.8 | 32.3 | 75.3 | 82.5 |
| | Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 73.0 mgm. per 100 cc. Volatile acids 0.55 cc. N/20 per 100 cc. | | | Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 57.08 mgm. per 100 cc. Volatile acids 0.55 cc. N/20 per 100 cc. | | |

TABLE 4—*Continued*

| PRODUCTS FORMED | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT MANNITOL | | | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT GLYCEROL | | |
|---|---|---|---|---|---|---|
| | Days incubated | | | Days incubated | | |
| | 2 | 6 | 9 | 4 | 7 | 9 |
| Total cubic centimeters of gas | 51.0 | 99.0 | 137.0 | 110.0 | 227.0 | 255.0 |
| Daily cubic centimeters of gas | 25.0 | 12.0 | 13.0 | 28.0 | 39.0 | 14.0 |
| Per cent of $CO_2$ | 65.3 | — | 85.0 | — | 46.5 | 45.4 |
| Per cent of $H_2$ | 27.2 | — | 10.8 | — | 44.5 | 4.8 |
| Per cent of residual gas | 7.5 | — | 4.2 | — | 9.0 | 11.8 |
| Cubic centimeters of $CO_2$ in gas | 33.0 | — | 116.0 | — | 106.0 | 116.0 |
| Daily $CO_2$ in gas | 16.0 | — | 27.0 | — | 35.0 | 5.0 |
| Cubic centimeters of $H_2$ in gas | 14.0 | — | 15.0 | — | 101.0 | 109.0 |
| Daily $H_2$ in gas | 7.0 | — | 0.3 | — | 34.0 | 4.5 |
| Ratio $H_2/CO_2$ | 1/2.4 | — | 1/7.8 | — | 1/1.0 | 1/1.0 |
| Ammonia-N milligrams per 100 cc. | 76.0 | 99.0 | 109.0 | 4.0 | 35.0 | 29.5 |
| Daily ammonia-N milligrams per 100 cc | 37.1 | 7.7 | 3.3 | 0.55 | 10.4 | −2.8 |
| Amino-acid-N milligrams per 100 cc | 113.79 | 116.36 | 66.80 | 73.74 | 92.81 | 86.51 |
| Daily amino-acid-N milligrams per 100 cc | 23.70 | 0.64 | −16.51 | 0.18 | 6.35 | −3.15 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc | 22.45 | 27.50 | 32.90 | 4.00 | 9.40 | 10.50 |
| Cubic centimeters of N/20 NaOH to neutralize volatile acids per 100 cc. daily | 10.90 | −1.26 | −1.80 | 0.86 | 1.80 | 0.55 |
| Per cent of volatile acids in fractions of distillate: 10 cc. | 6.7 | 5.2 | 4.6 | 2.5 | 5.3 | 6.9 |
| 20 cc. | 12.2 | 10.3 | 8.8 | 5.0 | 9.6 | 12.6 |
| 30 cc. | 16.9 | 15.1 | 12.8 | 6.3 | 12.8 | 17.4 |
| 40 cc. | 21.4 | 19.6 | 16.9 | 7.5 | 15.9 | 21.2 |
| 50 cc. | 25.4 | 23.6 | 19.1 | 17.5 | 19.3 | 25.0 |
| 100 cc. | 40.5 | 38.4 | 33.4 | 32.5 | 32.9 | 39.3 |
| 200 cc. | 58.8 | 57.3 | 52.9 | 45.4 | 58.0 | 55.5 |
| 300 cc. | 68.1 | 67.9 | 65.3 | 55.0 | 69.1 | 67.8 |
| 400 cc. | 74.8 | 75.4 | 73.5 | 62.5 | 76.6 | 74.8 |
| 500 cc. | 80.1 | 81.3 | 79.6 | 67.5 | 81.9 | 79.5 |
| | Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 66.39 mgm. per 100 cc. Volatile acids 0.65 cc. N/20 per 100 cc. | | | Before inoculation: Ammonia-N 1.8 mgm. per 100 cc. Amino-acid-N 73.02 mgm. per 100 cc. Volatile acids 0.55 cc. N/20 per 100 cc. | | |

TABLE 4—*Concluded*

| PRODUCTS FORMED | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT SOLUBLE STARCH | | | | ACTION ON 2 PER CENT PEPTONE SOLUTION, PLUS 1 PER CENT INULIN | | |
|---|---|---|---|---|---|---|---|
| | DAYS INCUBATED | | | | DAYS INCUBATED | | |
| | 4 | 6 | 9 | 12 | 4 | 6 | 10 |
| Total cubic centimeters of gas | 18.0 | 113.0 | 213.0 | 316.0 | 28.0 | 30.0 | 33.0 |
| Daily cubic centimeters of gas | 4.0 | 47.0 | 33.0 | 34.0 | 7.0 | 1.0 | 1.0 |
| Per cent of $CO_2$ | 44.1 | 73.6 | 71.2 | 79.3 | 20.6 | 29.3 | 42.4 |
| Per cent of $H_2$ | 53.6 | 23.5 | 24.7 | 18.0 | — | 70.0 | 50.6 |
| Per cent of residual gas | 2.3 | 2.9 | 4.1 | 2.7 | — | 0.7 | 7.0 |
| Cubic centimeters of $CO_2$ in gas | 8.0 | 83.0 | 151.0 | 251.0 | 6.0 | 9.0 | 14.0 |
| Daily $CO_2$ in gas | 2.0 | 38.0 | 23.0 | 33.0 | 1.5 | 1.5 | 1.5 |
| Cubic centimeters of $H_2$ in gas | 10.0 | 27.0 | 53.0 | 57.0 | — | 21.0 | 17.0 |
| Daily $H_2$ in gas | 2.5 | 8.0 | 5.0 | 1.0 | — | 10.0 | −1.0 |
| Ratio $H_2/CO_2$ | 1.2/1 | 1/3.1 | 1/2.9 | 1/4.4 | — | 2.4/1 | 1.2/1 |
| Ammonia-N milligrams per 100 cc | 40.0 | 50.0 | 52.5 | 81.0 | 47.5 | 60.0 | 69.0 |
| Daily ammonia-N milligrams per 100 cc | 9.5 | 5.0 | 0.8 | 9.5 | 11.4 | 6.3 | 2.2 |
| Amino-acids-N milligrams per 100 cc | 67.26 | 82.50 | 89.90 | 108.51 | 61.52 | 64.20 | 82.54 |
| Daily amino-acid-N milligrams per 100 cc | 0.68 | 7.62 | 2.43 | 6.20 | −0.75 | −1.34 | 4.58 |
| Cubic centimeters of N/20 to neutralize volatile acids per 100 cc | 3.50 | 16.15 | 23.95 | 31.65 | 1.10 | — | 11.9 |
| Cubic centimeters of N/20 to neutralize volatile acids per 100 cc. daily | 0.70 | 6.33 | 2.60 | 2.58 | 0.10 | — | 2.7 |
| Per cent of volatile acids in fractions of distillate: 10 cc. | 17.1 | 11.1 | 20.8 | 23.6 | 4.5 | — | 6.7 |
| 20 cc. | 31.2 | 20.8 | 39.9 | 41.8 | 9.1 | — | 13.4 |
| 30 cc. | 42.8 | 28.9 | 50.1 | 53.9 | 13.6 | — | 16.3 |
| 40 cc. | 54.3 | 35.4 | 58.2 | 60.0 | 22.7 | — | 24.4 |
| 50 cc. | 62.8 | 41.9 | 64.2 | 66.1 | 50.0 | — | 29.4 |
| 100 cc. | 88.5 | 63.0 | 90.2 | 96.4 | 68.2 | — | 51.2 |
| 200 cc. | 97.1 | 90.1 | 98.1 | 99.4 | 77.2 | — | 73.1 |
| 300 cc. | 99.9 | 97.1 | — | — | 81.8 | — | 88.2 |
| 400 cc. | — | — | — | — | — | — | 94.1 |
| 500 cc. | — | — | — | — | — | — | 97.4 |
| | Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 64.54 mgm. per 100 cc. Volatile acids 0.70 cc. N/20 per 100 cc. | | | | Before inoculation: Ammonia-N 2.0 mgm. per 100 cc. Amino-acid-N 64.54 mgm. per 100 cc. Volatile acids 0.70 cc. N/20 per 100 cc. | | |

Since there were such large amounts of ammonia present, and because it is so difficult to remove it from the sample, corrections by the use of the factors have probably given results as nearly correct as could have been obtained by any other method.

*Volatile fatty-acid determinations.* For the determination of volatile fatty-acids we have followed the Duclaux method as modified and described by Dyer (1916). This author suggested that the method might be employed in the study of acid production by bacteria, and Wolf and his associates used it for the study of the acid production by certain pathogenic anaerobes.

While the results which we have obtained show that there is a difference in the fermentative activities of this organism upon the different carbohydrates the differences are not great. The distillation of volatile fatty acids is not an easy task and while its use may be of value in detecting in a general way what products are formed during a given fermentation, their value as a diagnostic biochemical test for bacterial differentiation is limited.

Our attitude toward this test cannot be better expressed than by quoting from the summary of Wolf and Telfer.

> An experimental critique of the Dyer method of estimating volatile fatty acids has been made. This method, while perfectly satisfactory in the form stated by its author in dealing with a mixture of two volatile acids, the nature of which is known, fails when a mixture of unknown acids is to be analyzed. The color tests as proposed by him are satisfactory when dealing with pure acids, but not as positive as could be desired for the identification of an acid in the mixture. The separation of the acids is necessary before any reliance can be placed on these color tests.

From a study of table 4, it may be seen that in most cases the fractionation curves lie very near to those of propionic and butyric acids. By the tests devised by Dyer we could obtain no evidence of propionic acid, though various fractions were refractionated as indicated by Wolf and Telfer. We could obtain some fairly good tests for acetic and butyric acids after repeated refractionations; in the case of milk, the odor would indicate valerianic acid though no qualitative test could be obtained for this or for caproic acid.

The results indicated that the curve was due not to propionic acid but rather to a mixture of acetic, buytric and perhaps to valerianic acid. We felt, however, that work of this sort led to rather uncertain results, since we were able to simulate almost any acid curve desired by mixtures of other acids in the proper proportions.

The refractionation method proposed by Wolf and Telfer was not exhaustive enough to obtain various acids in a form sufficiently pure for accurate tests.

The fractional distillation of the cultures was completed in an attempt to find differences wide enough to be of diagnostic value, even though the acids present were not completely identified.

## DISCUSSION OF RESULTS

An examination of tab'e 4 will give an idea of the action of these orgainisms upon media rich, both in protein and carbohydrates. The cultures were very closely related in all respects in their biochemical reactions.

In most respects the quantitative results compare favorably with the qualitative results, but unfortunately do not give much additional information. Those showing large amounts of gas and acid in fermentation tubes, and Hiss serum water media containing the various carbohydrates and grown over phosphorus in an anaerobic jar, also show quantitatively larger amounts of gas and acid when grown in vacuum. It was impossible to obtain duplicates which checked exactly. Variation is inherent in all bacteriological work and the results obtained in this work check about as closely as those in many other bacteriological determinations.

*Probable error.* Two series of cultures were set up to determine the probable error. One series of five bottles containing two percent peptone-water after four days contained the following amounts of gas 92, 86, 61, 73, and 87 cc., respectively. A series of six bottles containing whole milk after two days contained the following amounts of gas 237, 243, 232, 203, 196, 186 cc., respectively. By using the formula:

$$E = 0.6745\sqrt{\frac{S}{N\,(N-1)}}$$

in which E = probable error, S = sum of residuals, N = number of parallel determinations. The probable error as determined for the first series is ±4.78, and for the second series is ±3.07.

The probable error was not determined for ammonia, amino-acid, or volatile-acid production, but duplicate determinations usually varied about as much as in the gas determinations.

*Nature of fermentation.* It will be noted that this organism produced considerable amounts of gas from peptone solution. This probably accounted for the presence of bubbles of gas in fermentation tubes containing non-fermentable carbohydrates. The higher negative tension and the larger amounts of media in the bottles tended to magnify the gas production from this medium in bottles, as compared to that in tubes in the anaerobic jar. (The latter usually showed a tension of about 350 mm. of mercury.)

When we mention the fact that an organism ferments a certain carbohydrate we usually refer to the fact that the change results in the production of gas or acid. These are products easily determined and are evidently due to deep-seated changes in the product. It is quite probable, however, that there may be organisms which bring about but slight changes in a carbohydrate, not extensive enough to lead to the formation of acid or even gas. It is also possible that these products may be produced and again utilized in the synthesis of cell protoplasm, or changed to other products of fermentation. It is a well known fact that the nitrifying bacteria utilize $CO_2$ as a source of carbon in the oxidation of ammonia and nitrites to nitrates. Nikitinsky (1907) showed that there were certain anaerobes which were able to utilize hydrogen. For this purpose he used "konzentrierten kanalisationsflussigkeit" and "Schlamm aus einem Absitzbecken" placed in an atmosphere of hydrogen. In one case he found that 500 grams of "Schlamm" was able to combine with an average of 30 cc. or a maximum of 70 cc. of hydrogen per day.

It is a well known fact that many bacteria can utilize acids and alcohols as a source of energy. It may also be possible that bacteria utilize only a minimum amount of the carbohydrate, enough to establish their initial development, but do not produce

changes, deep-seated enough to be detected by the ordinary chemical methods. After growth has been established they may then utilize some other element in the medium as their chief source of energy. It may be noted that the anaerobes studied by us grow very well in a solution of peptone and water.

These organisms could not utilize simple nitrogenous foods to any extent; nitrates, ammonia salts, urea and asparagin in a simple solution or in the presence of glucose give practically no development. They were able to grow well upon simple peptone solutions and attack the albumins of egg-white, blood serum, milk and meat, and also liquefy gelatin, either in the presence or absence of glucose. The changes in all cases are deep-seated since gas, volatile fatty acids, amino-acids and ammonia are formed from all of these media in fairly large amounts. Traces of hydrogen sulphide are also found in some of the meat cultures.

The proteolytic action is tryptic in nature, since it does not take place in a medium acid to litmus and is more active in a medium neutral to phenolphthaline than in one neutral to litmus.

*Gas production.* In cases in which large volumes of gas were present we considered that active fermentation had occurred, and this was correlated with the results of the fermentation tube tests. From these results, we are safe in considering that an anaerobe has attacked a carbohydrate vigorously only when it produces more than 1 per cent of gas in the closed arm of the fermentation tube, and a reaction distinctly acid to brom-thymol-blue. A bubble of gas might be taken to indicate slight fermentation of the carbohydrate, if it were present in the tube containing carbohydrate medium but not in a tube containing the media minus the carbohydrate. We have made as many as twenty parallel cultures of these anaerobes and treated all exactly alike; in some tubes there may be as much as one per cent of gas, in others a small bubble, and in others none at all, and all containing about an equal amount of growth. Since irregularities are unavoidable with these organisms, some arbitrary limit should be set. In this work we recorded a positive fermentation when more than one per cent of gas was formed and a questionable result when less than that amount was present if there were no marked change in the reaction.

In the case of lactose, inulin, arabinose and xylose there appears to be no doubt that these carbohydrates were not attacked to any extent. With mannitol there was an increase of gas and but little increase in acid above that in peptone solution alone. We have concluded that there was a slight fermentation of mannitol.

In most experiments the predominating gas was $CO_2$. Hydrogen was obtained in all cases except with the cultures upon asparagus at the beginning of the fermentation. One point of interest in this connection is the ratio of $H_2$ to $CO_2$. In all our tests the amount of $CO_2$ increased with the age of the fermentation, while the $H_2$ remained practically stationary after the first few days. This caused a change in the ratio of $H_2$ to $CO_2$ from day to day. Most authors mention a ratio between these gases as determined at a certain time. By determining it at intervals the ratio might be somewhat different as shown in this case. Apparently the ability of the organism to form hydrogen was limited to the beginning of fermentation; or the substance from which this gas was formed was used up in the earlier stages; or the hydrogen was oxidized as it was formed, as has been suggested by Nikitinsky, (1907) and Kaserer (1906).

According to Perdrix (1891) *B. amylomyze* produced a gas of variable composition with the different carbohydrates at different ages of the culture. He gives the following table to show this (for glucose).

| TIME | V | | R | D | |
|---|---|---|---|---|---|
| | Hydrogen | Ac. carb. | | Hydrogen | Ac. carb. |
| *days* | | | | | |
| 3 | 175 | 85 | 2.0 | 175 | 85 |
| 4 | 275 | 145 | 1.9 | 100 | 60 |
| 5 | 350 | 220 | 1.6 | 75 | 75 |
| 11 | 670 | 450 | 1.5 | 120 | 130 |

V = volume, R = ratio, D = difference.

Grimbert (1893) found that his *B. orthobutylicus* growing in glucose solution without chalk produced the following results:

| | HYDROGEN | AC. CARB. | R |
|---|---|---|---|
| Up to and including 4th day | 11.66 | 10.00 | 1.16 |
| 4th to 13th day | 11.24 | 32.76 | 0.34 |
| 13th to 22nd day | 1.90 | 6.90 | 0.28 |
| | 24.80 | 49.66 | 0.50 |

In our work the irregularities in the total amounts of the various gases were due to the fact that the amounts of gas were determined from the bottles which remained in the incubator for the longest time, and only part of these were analyzed at each period. An examination of the gas volumes as mentioned on page 398 will explain this variation.

In all cases there was considerable residual gas which could not be accounted for by the amount of air remaining in the bottles, since these were exhausted to nearly the theoretical vacuum. No doubt this was nitrogen gas which had been liberated from the medium. In no case was there more than a trace of $CO_2$ formed by combustion with oxygen. For this reason we may say that no methane or ethane were formed by these organisms.

It will be noticed from table 4, that gas is produced slowly in all cases except in presence of glucose and glycerol. These substances are easily fermented and all cultures produce gas in large amounts. In the presence of lactose, there is about the same action as upon peptone solution except that slightly more hydrogen is produced in some cases, and the growth is slightly more vigorous. A slight action upon the lactose may have occurred in the latter case.

*Ammonia.* The ammonia production is not always in inverse ratio to the production of gas and acid by these organisms. The cultures showing larger amounts of gas show smaller amounts of ammonia in some cases but not in all. Glucose reduced the ammonia production for this organism. In the presence of lactose, mannitol and sucrose there was practically no reduction in the ammonia formation. In the presence of salicin there was a decrease, and the organism is to be considered a salicin fermenting type. Peptone solution plus glycerol shows much less ammonia

produced and there was clear evidence that the organisms attacked the glycerol. There seemed to be vigorous growth in inulin-peptone solution but the action upon the medium was not so vigorous.

The proteolytic action of these organisms is also shown by the production of ammonia from asparagus, alkaline egg medium and whole milk.

*Amino-acids.* In the liberation of amino-acids from the peptone solution plus carbohydrates there is a correlation similar to that seen in the production of ammonia from similar solutions. From peptone alone there is a marked decrease in the amino-acid content after the first three days.

The freeing of amino-acids does not correspond to the ease with which a carbohydrate is fermented. There is an increase in the amino-acid content for a certain period in all cultures. In the presence of glucose, salicin, sucrose, starch and inulin this increase continues to the close of the experiment. With lactose and glycerol there is a slight decrease as the culture ages. In case of mannitol there is a decrease from the beginning. There is a marked production of amino acids from asparagus, alkaline egg medium, and whole milk. In a purely nitrogenous media there is a marked increase in the amino-acid nitrogen, followed by a decrease as seen in alkaline egg and peptone solutions. This point is not so marked in media containing fermentable compounds such as asparagus, milk, glucose, salicine, glycerol and starch solutions. Apparently the organisms may not attack the amino-acids as readily in the presence of fermentable carbohydrates as they do in their absence, or they may be produced more rapidly than they are used and thus accumulate in the medium. It is impossible to elucidate this point at present since total nitrogen determinations were not made on each sample at various periods.

*Volatile fatty acids.* Fairly large amounts of volatile fatty acids were produced in most cases. The figures in table 4 represent cubic centimeters of N/20 alkali used to neutralize the acid obtained by complete distillation, as above described. The per cent of volatile acid in each fraction was obtained by dividing the amount by the total acid. Usually there was a change in the

total amount of volatile acid, and in most instances some change in the fractionation constant from day to day. With peptone alone, there was little change, but with glucose, salicin, glycerol, and soluble starch, there was an increase, while mannitol caused a decrease. These differences were constant and are probably due to a slight difference in the fermentation reaction which occurred in these cultures at different ages. The increase in the amount of acid obtained in the first fractions would indicate an increase in the higher acids; a decrease in the amount of acid obtained in the first fractions would indicate an increase in the lower acids over those originally present.

Less acid was produced in the presence of glycerol and soluble starch than was produced from peptone alone. Apparently the gas and acid production are not always related in the fermentation of the various carbohydrates. This is shown to be especially true of glycerol and soluble starch in which there are very large amounts of gas and only small amounts of acid formed. The distillation curves for glucose, glycerol and inulin indicate the presence of an acid of low molecular weight, probably acetic acid; while curves obtained from starch indicate large amounts of acids of high molecular weight, probably butyric. Usually the same type of curve was obtained throughout the entire incubation period, indicating that the difference in the gas ratio is not closely correlated to the production of different kinds of acid. Apparently the $CO_2$ in the later stages of fermentation comes from the amino-acids present and not from the carbohydrates.

## CONCLUSIONS

1. We have described a method adapted for the quantitative study of the decomposition products formed by anaerobic bacteria.

2. The method described has been found very satisfactory for the cultivation of anaerobic bacteria. It has also proven very satisfactory as a means of measuring the amount and kind of gas produced.

3. The objection to the use of such an apparatus is that it does not give the anaerobes an oxygen tension which might be more favorable to their development. Even the "obligate" anaerobes

probably utilize a certain amount of free oxygen when it is available.

4. The quantitative results obtained in this particular case do not happen to yield results of significance in the subdivision of the group of organisms studied. They do, however, give us a clear picture of the biochemical behavior of these types which will make possible an accurate comparison of their physiology with that of other forms which may later be investigated.

5. It may be hoped that chemists will interest themselves in the improvement and simplification of chemical methods necessary to determine quantitatively and biochemically changes due to the growth of microcrganisms in various culture media. As such methods are devised the bacteriologist will obtain an increasingly sound and logical basis for the differentiation of microbial species, and will obtain much valuable information concerning the nature of the life processes of microörganisms.

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# THE PROPORTION OF VIABLE BACTERIA IN YOUNG CULTURES WITH ESPECIAL REFERENCE TO THE TECHNIQUE EMPLOYED IN COUNTING

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## PART I

During the course of some work on the morphological changes occurring during the life history of certain bacteria, it became desirable to ascertain the true relationship existing between the number of living organisms and the total number of cells in a young broth culture. A few preliminary experiments were sufficient to show that a certain discrepancy existed between these numbers in spite of the fact that it has been frequently stated, and in many cases taken for granted, that in a young broth culture —up to twenty-four hours—all the bacilli are viable. On looking up the literature it was found that of the many observers who had made a comparison of the two counts—Hehewerth (1901), Zelikow (1906), Eijkman (1904), Winterberg (1898), Klein (1902), Wright (1902), Anderson, Fred and Peterson (1920) and Glynn, Powell, Rees and Cox (1913)—not one had succeeded in proving the two to be identical. In fact, in the majority of cases, a definite numerical inferiority was found in the case of the viable count. In nearly every case this discrepancy was passed over with but little comment, the results being explained by errors in the technical procedure adopted. The argument appears to have been that because in the early stage of a bacterial culture all the bacilli are living, then any failure in the agreement of the total and viable counts must be due to technical error. To determine

this point exactly it was clear that a technique would have to be evolved which would permit of as small an error as possible.

A careful study of the literature seemed to show that few of the methods which have been employed hitherto could claim to conform to such a degree of accuracy. Generally speaking, the methods which have been employed may be classified into (1) the direct and (2) the indirect. In the former the organisms are counted directly under the microscope, in the latter the number of bacteria present is calculated from an enumeration of the colonies which develop when an aliquot part of the emulsion in question is mixed with a nutrient medium in a Petri dish, and incubated for a variable period of time. The former is designed to record the total number of organisms present, the latter only the number which happens to be viable at the moment of sampling.

With regard to the direct or total count, Klein (1900) appears to have been one of the first to realize the value of such a method of estimation. His technique consisted in staining a moist preparation of organisms with aniline gentian violet, spreading a loopful of known capacity on a coverslip, drying, and clearing in xylol balsam. The total number of fields on the coverslip was determined for a definite combination of lenses, fifty fields were counted and the number of stainable organisms per cubic centimeter in the original culture was calculated. This technique was followed by Hehewerth and Eijkman, and in a somewhat modified form by Anderson, Fred and Peterson. Zelikow introduced a method whereby the number of bacteria was determined by estimating the amount of dye they were able to adsorb from a solution of fuchsin, the strength of the latter before and after adsorption being determined by means of a Duboscq colorimeter. Winterberg conducted his count in a Thoma-Zeiss chamber. Wright's method of counting against red blood cells is too well known to need description. A modification of it was described by Harrison (1905) who observed the mixture of bacteria and red cells in a moist film instead of in the dried condition. Finally, a new form of counting chamber—the Helbe—similar to the Thoma-Zeiss, but measuring only 0.02 mm., in depth, and fitted with an opti-

cally plane cemented coverslip for use with a $\frac{1}{12}$ inch objective, was employed by Glynn, Powell, Rees and Cox, the organisms. being examined in the stained condition under open illumination.

The indirect or viable count has, as a rule, been performed by a modification of Koch's original plating method. The only exceptions which need be noted are those of Naegeli and Schwendener (1877) who took the amount of fermentation as their basis for the computation of the number of growing organisms, and Winterberg who accepted motility as his criterion of viability. The modifications of Koch's method have been concerned with the medium used, the question of preliminary dilution, the methods of dilution and the exact technique of counting the plates. The majority of observers appear to have used agar, but Buchner, Longard and Riedlin (1887), Hehewerth, Eijkman, Zelikow, and Chick (1912), seem to have preferred gelatin, though in some cases both media were employed. With regard to preliminary dilution, the earlier workers generally preferred to plate out the original emulsion, while of late the tendency has been in the opposite direction, as in the case of Winterberg, Zelikow, Müller (1895), Rahn (1906), Madsen and Nyman (1907), Lane Claypon (1909), Penfold (1914), Coplans (1909), Chesney (1916), and Noyes and Voigt (1916). The method of dilution has been subject to considerable variation; on the whole volumetric pipettes have been most popular, but Lane Claypon, Ficker (1898), Chick, and Penfold used dropping pipettes, while Hehewerth, and Graham Smith (1920) resorted to the use of a standard platinum loop. Gotschlich and Weigang (1895) elaborated a special technique in which dilution was performed by a combination of gravimetric and volumetric methods. The important question of the counting of the plates has naturally depended largely on whether or no a preliminary dilution of the emulsion was made. Where the number of colonies was very great, microscopic counting was adopted, usually with the aid of a Wolfhügel's plate, as in the case of Buchner, Longard and Riedlin, Where on the contrary, dilution was employed, the use of the microscope was no longer necessary, and counting was performed with the naked eye or with a magnifying glass—Krönig and Paul,

Zelikow, Neisser, Lane Claypon, Penfold, and Moore (1915). Graham Smith used a dissecting microscope, while Frost (1921) has evolved a microplate method for purposes of rapid enumeration of organisms in milk. The foreging summary of the literature is only intended to indicate the main outlines which have been followed, a complete survey lying beyond the scope of this paper.

In perusing the results of previous workers, it was striking to observe the peculiar lack of attention which was paid to the estimation of the experimental error involved in the methods employed. Probably this is to be attributed to the fact that in many cases in which the enumeration of bacteria was undertaken, a relative, rather than an absolute accuracy was essential. It was felt that the successful accomplishment of this object could only be attained by working out a technique in which the errors inherent in every step should be known with certainty. The fulfillment of this requirement has been kept constantly in mind throughout the course of the present work.

### *Technique of the total count*

After careful consideration and personal experience of several methods—especially those of Wright and of Brown and Kirwan (1915)—it was decided to adopt the method which has been in use in this laboratory during the past two years. Practically, this resolves itself into the adaptation of the Helbe counting chamber to dark-ground illumination. Instead of examining the organisms in a stained condition with open illumination they are observed in their natural state against a dark background. For use with a parabolloid condenser, the only alteration of the chamber which is required, is a selection of a slide of such a thickness that the distance between the lower surface of the chamber and the upper surface of the condenser shall lie between 0.9 and 1.1 mm. With a slide of other dimensions than these the correct focussing of the rays of light becomes impracticable. The depth of the chamber is 0.02 mm., while the surface is ruled into small squares.[1] The best combination of lenses has been found to be

[1] A slide of the dimensions quoted was made by Messrs. Hawksley & Son.

a two-thirds objective and an eighteen compensating ocular. Preliminary dilution of the emulsion is made—where necessary—by means of dropping pipettes—to be afterwards described—the diluent used being a 1 per cent solution of phenol in 0.9 per cent saline. If the preparation is examined immediately after adjustment of the coverslip, the bacilli stand out as light, refractile rods having a yellowish color, and showing a certain amount of Brownian movement; after a lapse of ten or fifteen minutes, the bacilli begin to settle on the floor of the chamber and of course lose their molecular motion; though in this condition they are quite easy to recognize, it is better to examine the preparation directly after it is put up so as to have the advantage of the Brownian movement, the degree of which was soon recognized to be characteristic of bacilli, as opposed to that possessed by minute particles of albumins and other material which is of a far more active nature.

The only serious drawback that has been encountered in the use of this method is that it is unsuitable for dealing with broth emulsions containing fewer than fifty million organisms per cubic centimeter. This objection is generally of little importance, for the majority of counts one desires to make are concerned with much thicker emulsions, but the force of the disadvantage became clear when we wished to perform total counts on the early stages of a young broth culture before any visible turbidity had appeared. For this purpose we at first resorted to the use of a Thoma-Zeiss slide of 0.01 mm. depth, the bacilli being suspended in a weak solution of methylene blue, and examined by open illumination. Experience showed, however, that such a method was unreliable, errors of 50 per cent in comparative counts being met with; it was therefore abandoned altogether. So far, no satisfactory solution of this difficulty has been found.

### *Estimate of the error of the total count*

Throughout most of the work to be described in this paper, a particular strain of *Bact. suipestifer* has been used, but for some of the earlier counts the stock strain of *Bact. typhosum* was selected.

In order to ascertain the error involved in the actual process of counting, three experiments were performed on three separate occasions. On the first occasion an emulsion of *Bact. suipestifer* in 1 per cent phenolised saline was counted eight times in succession, and the results compared. They are shown in table 1, the counts being in terms of the original emulsion.

On the second occasion, using an emulsion of *Bact. typhosum*, five counts were made, with a resulting percentage mean error of 4.04 per cent. On the third occasion ten counts were performed with a resulting percentage mean error of 4.95 per cent.

TABLE 1

| NUMBER OF COUNT | COUNT PER CUBIC CENTIMETER |
|---|---|
| 1 | 13,830,000,000 |
| 2 | 14,400,000,000 |
| 3 | 15,130,000,000 |
| 4 | 14,730,000,000 |
| 5 | 15,830,000,000 |
| 6 | 14,100,000,000 |
| 7 | 15,000,000,000 |
| 8 | 14,830,000,000 |
| Arithmetic mean | 14,730,000,000 |
| Mean error | 464,000,000 |
| Percentage mean error | 3.15 |

From these three experiments it is seen that the mean error of performing the total count under the conditions described may be taken to be not greater than 5 per cent. It is interesting to compare these results with those obtained by Glynn, Powell, Rees and Cox. Working with staphylococci, streptococci, and *Bact. coli* they found that in the case of an 0.02 mm., chamber the average percentage deviation from the arithmetic mean was 3.1 per cent; with an 0.1 mm., chamber it was 5.4 per cent, and with Wright's blood film method it was 34.1 per cent. With regard to Klein's method, Gotschlich (1912) quotes the mean error of the total count as 19 per cent.

### *The method of dilution*

Before proceeding further it will be necessary to give details of the methods of dilution which have been employed in both the total and the viable counts. For this purpose dropping pipettes have been preferred to volumetric pipettes, chiefly because in following out the growth of a bacterial culture such a large number of volumetric pipettes are required that the price of the latter becomes prohibitive: moreover dropping pipettes are more convenient to handle and more accurate in their delivery.

For full details with regard to the preparation of these pipettes and for an account of the precautions to be observed in handling them, the original article by Donald (1915) should be consulted. Suffice it to say that his technique has been used throughout with the exception of one or two modifications. Thus, for the purpose of calibration, an Imperial Standard wire gauge has been employed and, in order to insure accuracy, the pipettes have been subsequently tested by means of an ordinary screw micrometer measuring to 0.01 mm. Further, the drop values have been estimated by gravimetric, instead of by volumetric methods. The actual size selected was no. 22 on the wire gauge. After working out the drop volumes of various fluids at the temperature at which they were to be used, one factor remained to be determined, namely the effect of variations in density of a bacterial emulsion on the size of the drop delivered. On investigating this point, it was found, contrary to expectation, that there was no appreciable difference in the drop values of emulsions ranging in thickness from 500,000,000 to 10,000,000,000 organisms per cubic centimeter. The explanation of this would appear to be that as the emulsion increases in density, the weight of the organisms in the drop just suffices to counterbalance the increasing viscosity of the liquid, so that the actual value of the drop remains approximately constant.

### *Errors of the dropping pipettes*

In order to determine the errors of the dropping pipette a considerable number of experiments were made, the details of which

it is not proposed to enter into here as the space required would be greater than would be warranted by their relative importance. The results arrived at may be set out in the following order: (1) In making any dilution not less than four drops should be used, as with a smaller number sufficient accuracy is difficult to obtain. With four drops, however, or more, the error of delivery does not exceed 1.2 per cent. (2) The bacterial content of drops delivered in succession appears to be uniform; no difference between individual drops could be substantiated. (3) So long as the interior of the pipettes is clean--and all pipettes should be washed through with alcohol and ether—only a comparatively small number of bacteria remains adherent to the walls; this error of clinging does not appear to be greater than 1 per cent. (4) In making serial dilutions a separate pipette should be used for each emulsion. Under these circumstances the deviation of the actual from the calculated dilution was found to be not greater than 3 per cent.

*The employment of roll tubes for the estimation of the viable count*

Before entering on a complete description of the method employed in the estimation of the viable count, it is thought advisable to give a brief resumé of some of the technical details which had to be worked out in order to insure the greatest degree of accuracy. In the first place Petri dishes have been discarded in favor of roll tubes. These are prepared in the following manner: Test tubes measuring 6 inches by $\frac{5}{8}$ inch are selected; after sterilization in the hot oven, about 2 cc., of nutrient agar is placed in each. They are then autoclaved for twenty minutes at 120°C. When required for use the agar is melted and allowed to cool to 45°C., in a water bath; the inoculum is delivered directly into the tube, the contents of which are mixed by gentle shaking. The tube is then rolled between the fingers—as in the case of the Esmarch roll tube—the agar being allowed to pass about halfway up the tube. As a rule solidification is complete in half to one minute, and the tubes are then incubated in an inverted position for three days at 37°C. At the end of that time, when they are removed, it will be found that the agar is studded with colonies varying from round to lenticular in shape, evenly distributed

throughout the tube. In order to count the number which has developed, a series of circles, 1 cm. apart, is lightly drawn around the tube with an oil-marking pencil, and a longitudinal line drawn from top to bottom intersecting each circle at right angles. The actual counting is performed by means of a small magnifying glass, the tube being examined against a dark back-ground illuminated by a partially concealed electric bulb. The contents of each circle are observed in turn from above downwards, till the total number of colonies in the tube has been enumerated. This procedure is greatly facilitated by the registration of each colony on a small counting machine—a process which has the double advantage of not only saving one the trouble of memorizing the figures, but also of eliminating the personal factor in counting, for one has no idea of the actual number till the tube has been completely examined.

In using roll tubes for this purpose one or two small points should be noticed. The temperature of the agar at the time of inoculation may be allowed to vary between 41° and 50°C.; no deleterious effect of a temperature even as high as 55°C. could be noticed in experiments made to determine this point. Further, it is well to allow the agar tubes after melting to remain in a water bath at 45°C. for five to ten minutes, so as to allow of the condensation of some of the excess moisture suspended in the air of the tube, otherwise it may be deposited on the surface of the agar after rolling, and lead to an undesirable amount of spreading. It is to allow any such moisture to run down on to the cotton plug and so be absorbed that the tubes are incubated in an inverted position. Finally to prevent excessive condensation, it is advisable to place the tubes in the incubator as soon after rolling as possible.

### *Comparison of tubes and plates*

The question will naturally arise as to the reason for the abandonment of plates in favor of tubes. In reply the following factors may be adduced: (1) The prohibitive cost of plates when large numbers are required for use. In many experiments 40 and 50 tubes have been used at a time, and as these experiments

have been repeated from day to day, the acquirement of a stock of 200 or 300 plates would have been necessitated, had these been employed. (2) There is considerably less risk of contamination in the case of tubes. Absolute sterility is obtained by autoclaving the agar. Contamination, in fact, does not occur. In dealing with several thousand tubes during the course of this work, only one single case of contamination has been encountered. With plates, on the contrary, the risk of contamination is by no means negligible, especially if incubation has to be continued for three days. (3) Less media is required in the case of tubes than with plates; for the former 2 cc. are sufficient, for the latter—if a 5-inch plate be employed—16 cc. or so must be prepared. (4) Tubes are easier to count than plates. They possess no corners; one is dealing with a relatively flat surface throughout, whereas with plates there is frequently an undesirable amount of spreading of the colonies at the junction of the bottom with the sides which renders counting a difficult and uncertain procedure. (5) Tubes can be incubated immediately after rolling, instead of having to be kept immobile for twenty minutes till the agar is set, as in the case of plates. Where time is a consideration the practical value of this point will be appreciated.

After finding that tubes were perfectly well suited for the purpose of performing the viable count, it was necessary to compare the results obtained with those obtained by means of plates. For this purpose two distinct sets of experiments were undertaken, differing in the way in which the plates were poured. In the first series viable counts were made on various broth cultures employing both tubes and plates. The tubes were put up and rolled in the way already described. In preparing the plates the emulsion to be counted was delivered into a test tube containing 15 cc. of melted agar which was then poured into a plate and allowed to solidify. Each was incubated for three days before counting. In all cases the conditions were strictly comparable. During the course of several experiments of this nature 69 tubes and 69 plates were examined. The total number of colonies in each set were added together: the results were as follows:

| NUMBER OF COLONIES IN TUBES | NUMBER OF COLONIES IN PLATES | PERCENTAGE OF COLONIES IN PLATES TO THOSE IN TUBES |
|---|---|---|
| | | *per cent* |
| 8741 | 7527 | 86.1 |

In reviewing these figures one was naturally struck by the distinct numerical inferiority of the plate colonies. It was surmised that part, at least, of this discrepancy might be attributable to the method of pouring, since in this procedure a certain unknown quantity of agar is bound to be left behind in the tube. To test this a second series of experiments was carried out, similar to the first, except that the drops of emulsion were delivered directly into the plate, and the agar poured on top of them, mixing being performed as well as possible by gentle tipping to and fro of the plate. In the course of three experiments a comparison of 14 tubes and 14 plates was made, with the following results:

| NUMBER OF COLONIES IN TUBES | NUMBER OF COLONIES IN PLATES | PERCENTAGE OF COLONIES IN PLATES TO THOSE IN TUBES |
|---|---|---|
| | | *per cent* |
| 8041 | 7636 | 94.93 |

Comparing these two sets of experiments it would appear that approximately 9 per cent of the contents of the tubes must be left behind when these are used for pouring agar into the plates. This is somewhat higher than the figure quoted by Winterberg, who likewise estimated this error, though in a different way: he found it to be about 5 per cent. At any rate it may be concluded that this method of inoculating plates does introduce a considerable error, the effect of which will be to give a uniformly lower count than should actually be the case. On the other hand it would be thought that when the emulsion is delivered directly into the plate, and the agar poured over it, the count should be the same as that yielded by the roll tube method; it is seen, however, from the second protocol that the plate count is still 5 per cent below the roll tube count. The explanation of this may lie in the possibility that too small a number of experiments was

made, but, while granting that this may be true, it would appear more probable that the difference is due chiefly to a lack of uniform mixing of the emulsion with the agar in the plate preparations. An examination of these showed that the colonies tended to be aggregated in groups, while in the roll tubes they were distributed equally throughout the agar. The effect of this crowding is, as is afterwards demonstrated, to lead to the failure of certain bacilli to develop into single colonies; hence a smaller number of colonies will result than would otherwise be the case when the bacilli are separated by a sufficient space from each other. If, in the plate preparations, uniform mixing could be insured, this difference would probably disappear; incidentally, however, the difficulty of insuring such a mixture is by no means imaginary, and whatever method be employed for gaining this purpose an unnecessarily large amount of time must be spent over each plate.

To sum up briefly, then, it is claimed that roll tubes possess certain advantages over plates, the chief of which is undoubtedly that the count tends to be higher—probably at least 5 per cent—and therefore presumably more accurate. The only objection to their use is that they take a slightly longer time to put up; this is more than counterbalanced, however, by the greater rapidity and ease with which they can be counted.

### *The nature of the diluent*

For counting emulsions dilution has been preferred to direct sowing. For this purpose a suitable number of drops is delivered into a flask containing the diluent, and if necessary a further dilution is made by the use of a second flask. In every case a fresh pipette is used for each dilution.

In the early stages of this work attention was directed to the nature of the diluent used in preparing the emulsions for counting. A review of the literature shows that the most popular fluids employed for this purpose have been saline, distilled water, and tap water. A preliminary experiment undertaken to ascertain whether any material difference could be discerned between these

three diluents showed that the organisms remained alive longest in tap water, while in distilled water they rapidly died out. With regard to saline, Flexner (1907) found that a pure solution of sodium chloride was distinctly inimical to the life of the Meningococcus. More recently Shearer (1919), while confirming this result, pointed out that the deleterious action of this salt could to a certain extent be neutralized by the addition of a small amount of calcium.

To investigate this effect more closely, a saline emulsion of a five and one-half hours' broth culture of *Bact. suipestifer* was prepared, and roll tubes were put up from it at various intervals, 3 to 5 tubes being employed for each count. The emulsion was put up at 3 p.m., and the counts were made each hour till 6 p.m., and again the following morning at 9.30 a.m. The results were as follows:

TABLE 2

| TIME | COUNT PER CUBIC CENTIMETER |
|---|---|
| 3 p.m. | 125,500,000 |
| 4 p.m. | 120,400,000 |
| 5 p.m. | 101,100,000 |
| 6 p.m. | 45,380,000 |
| 9.30 a.m. | Sterile |

"Sterile" = no organisms developing in a tube seeded with 0.1 cc. of the emulsion.

The effect, then, of eighteen and one-half hours' contact of the bacilli with saline, was to sterilize the emulsion. Considering that this effect might be due to absence of certain salts in the diluent which were necessary for the continued viability of the organisms, comparisons were instituted between tap water, saline, and Ringer's solution. Three sets of two flasks were taken, and equal quantities of these three fluids placed in them, respectively, in this order. The primary and secondary dilutions were made as nearly simultaneously as possible. After performing a viable count on each of these secondary dilutions, the flasks were lightly plugged with cotton wool to protect them from contaminating organisms. Viable counts were made at intervals up to twenty-

one hours on each of them. The results have been plotted in the form of a graph to render them more striking (fig. 1). The figures are given in terms of the original culture. From this it will be seen that while all three fluids have a deleterious effect on the organisms, that of Ringer's solution is the least marked, and saline the most pronounced, that of tap water occupying an intermediate position.

A fresh experiment was performed with the idea of discovering what proportion of Ringer's solution was necessary to prevent

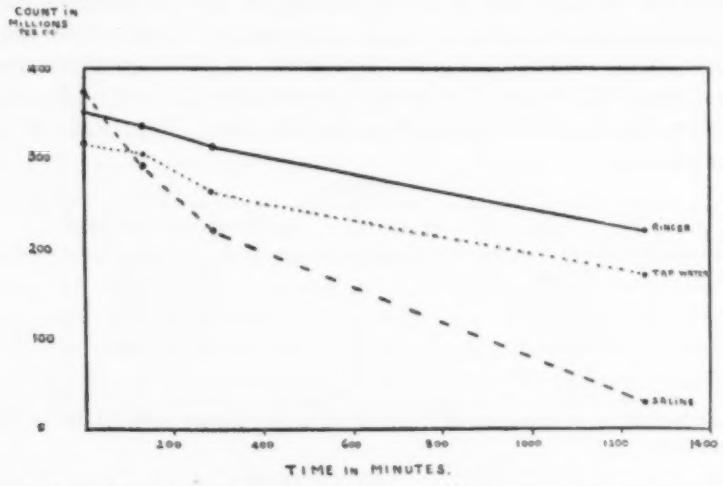


FIG. 1. SHOWING THE EFFECT OF VARIOUS DILUENTS ON THE VIABILITY OF BACTERIA

the deleterious effect of distilled water on organisms emulsified in it. A quantity of Ringer's solution was prepared according to the following formula:

| | | |
|---|---|---|
| Sodium chloride | 0.9 | grams |
| Potassium chloride | 0.042 | gram |
| Calcium chloride | 0.048 | grams |
| Sodium bicarbonate | 0.02 | gram |
| Distilled water | 100.0 | cc. |

From it five separate dilutions were made with distilled water, commencing with one-half and passing by geometrical progres-

sion up to one in thirty-two. A three and one-fourth hour broth culture at 37°C. of *Bact. suipestifer* was used for counting and dilutions were put up almost simultaneously in the six fluids at hand. Viable counts were conducted at the start and thereafterwards at intervals up to 49 hours. In all cases the counts were performed under strictly comparable conditions. The results are shown in table 3, the counts being given in terms of the original culture.

Neglecting the slight irregularities in the series, which were probably due to small differences in the dropping pipettes used—this experiment being performed before a micrometer had been obtained for checking their calibration—it is at once clear that

TABLE 3

| TIME | RINGER CONCENTRATION | | | | | |
|---|---|---|---|---|---|---|
| | Pure | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| *hours* | | | | | | |
| 0 | 109,200,000 | 112,800,000 | 101,900,000 | 111,700,000 | 98,830,000 | 87,770,000 |
| 3½ | 94,190,000 | 91,680,000 | 101,200,000 | 66,720,000 | 72,430,000 | 1,962,000 |
| 21 | 27,830,000 | 27,650,000 | 28,010,000 | 27,110,000 | 31,930,000 | Sterile |
| 27¼ | 28,910,000 | 19,270,000 | 9,633,000 | 12,490,000 | 19,630,000 | Sterile |
| 48½ | 15,340,000 | 2,855,000 | 1,427,000 | 2,141,000 | 3,925,000 | Sterile |

up to twenty-one hours the 1:16 dilution of Ringer's solution is no more harmful to the bacilli than the pure solution. But between the 1:16 and the 1:32 dilution there is an enormous difference; the latter acts just like distilled water, rendering the emulsion sterile within twenty-four hours. From this it may be inferred that a minute but quite definite amount of various kinds of salts is necessary to prevent the deleterious action of distilled water. What particular quantities of what particular salts was not entered into more fully, as it lay outside the scope of this work. The practical conclusion to be drawn from these experiments seems to be that in preparing dilutions for a viable count on a bacterial culture, the best fluid to use, so far investigated, is undoubtedly Ringer's solution; particularly is this the case if the emulsions have to be kept for some time, or possibly shaken, before the actual agar is inoculated. If the tubes are to be put

up within five or ten minutes of preparation of the dilutions, it is immaterial whether tap water, saline, or Ringer's solution be employed. For most purposes in this work tap water, sterilized by autoclaving at 120°C. for forty-five minutes, has been chosen as the diluent, and been found perfectly satisfactory. Just recently however, during the drought of June and July, it was noticed that the three tubes put up for each count during the course of routine work showed a marked disagreement with one another, and, on investigation this was referred to the tap water. Whether any marked change was produced in the saline content as a result of the drought, or whether possibly some metallic contamination had gained entrance to the water, was not ascertained, but on replacing the tap water by Ringer's solution, the tubes again showed close agreement with one another.

*Nature of the medium*

The nutrient agar used was prepared from a three weeks' casein digest at 37°C. (Cole and Jordan Lloyd, 1917). After filtration it was diluted till it had an amino-nitrogen content—as determined by Sörensen's method of formol titration—of 0.08 per cent, this being found to be the most suitable proportion for the purpose. An addition of 2.5 per cent agar was then made—higher amounts being found to exert a deleterious action on the development of the organisms. As no advantage of washed over commercial agar could be substantiated for the growth of *Bact. suipestifer*—a result which failed to confirm the findings of Ayers, Mudge and Rupp (1920) in the case of milk bacteria—the latter was uniformly employed. The hydrogen-ion concentration of the finished medium was allowed to vary from pH 7.4 to pH 7.8.

*Length of incubation and effect of moisture during incubation*

With regard to the length of time that the tubes should be incubated, experiments were made in which the number of colonies was counted on successive days up to a week. The result was to show that though the majority of the bacilli grow within the first twenty-four hours at 37°C., a few colonies

do not appear till the second or third days after inoculation. Later than this no further numerical increase was observed.

*The effect of shaking on broth cultures*

In view of the fact that the discrepancy in the relation of the viable to the total count has frequently been attributed to clumping of the organisms, leading to the development of a single colony from one mass of organisms instead of to several, and in view of the fact that the aim in this work was to eliminate every factor hindering the development of the maximum number of colonies, it was decided to investigate the effect of shaking on the cultures before the counts were put up. Shaken and unshaken cultures were compared, both total and viable counts being performed on them, the conditions being rendered identical except for the single process of shaking. As a result of several experiments on broth cultures of different ages it appeared that shaking in the case of a broth culture under twelve hours old led to no increase in either count; in a twenty-four hour culture, however, an increase of somewhere in the neighbourhood of 15 to 25 per cent could be substantiated in both. In the estimation of the count in young cultures, therefore, shaking is of no value: for the estimation of the count in older cultures, shaking leads to an absolute increase in both total and viable counts, but as the relation between the two remains unaltered, it is of no advantage so long as it is only this ratio which is being investigated.

*The optimum number of colonies per tube*

Speaking of the technique of the plate count Park and Williams (1908) remark:

> It is very important to remember that when more than 200–300 bacteria start to develop in the agar or gelatin contained in a plate some develop colonies which fuse together, while others are inhibited before they develop visible colonies. . . . . If possible, dilutions should be made so that plates will contain between 40 and 400 colonies.

This is one of the few references I have been able to find to the

effect of overcrowding in the determination of the numerical development of colonies in plates. However, in considering this question it must be realized that two factors are concerned. The first may be called the error of sampling, the second the error of overcrowding.

*a. The error of sampling.* It is clear that if three tubes are put up from an emulsion containing a comparatively small number of bacilli, the chances of obtaining a representative sample must be smaller than if an emulsion be employed which contains a much larger number of bacilli. Similarly with the tubes themselves. If only a few bacilli are introduced, the chances of obtaining a correct idea of the exact number are smaller than if a large number of bacilli are introduced. Thus the greater the number of colonies per tube, the less is the error of sampling. That this is not a mere theoretical consideration is shown from an examination of the data accumulated during the progress of his work. Thus to quote from the actual figures of an experiment. Three tubes were put up from a particular emulsion: in the first, 7; in the second, 12; and in the third, 15 colonies developed. The arithmetic mean of these three is 11.33, and the percentage mean deviation is 25.5 per cent. Or again; in a series of three tubes inoculated from the same dilution, the number of colonies developing was 434, 454 and 470. The arithmetic mean of these is 452.7, and the percentage mean deviation is 2.75 per cent. From these two examples it is seen that the percentage deviation of each individual tube from the arithmetic mean was considerably greater in the case when a small number of colonies developed than in the case when a large number of bacilli were inoculated. In other words the sampling error in the former instance was large, in the latter comparatively small. Recognizing however, the fallacies of a single experiment, a large number of experiments were reviewed in which viable counts were put up by means of three tubes each; the arithmetic mean of each of these counts was worked out, and the percentage deviation of each count from the arithmetic mean estimated. The percentage mean deviation was calculated for tubes in which 0 to 50 colonies developed, and again for those in which 50 to 100, 100 to 200, 200 to 400, 400 to

800, and 800 and over developed. The results are shown in table 4.

These figures, of course, do not represent the sampling error itself; this can only be obtained by putting up comparable series of tubes from the same emulsion; but they do show that the fewer colonies there are per tube, the less chance is there of obtaining an accurate sample of the emulsion under investigation. When more than 800 develop, the increasing accuracy of representation is overshadowed by the error introduced by the difficulty experienced in the actual counting of the tubes. The conclusion may therefore be drawn that in order to keep the sampling error as low as possible, somewhere between 400 and 800 bacilli should be inoculated per tube. Naturally, if more than three tubes are put up, the sampling error will be smaller, but as

TABLE 4

| | 0 TO 50 | 50 TO 100 | 100 TO 200 | 200 TO 400 | 400 TO 800 | 800 AND OVER |
|---|---|---|---|---|---|---|
| Number of experiments | 13 | 24 | 43 | 42 | 42 | 11 |
| Percentage mean deviation | 19.6 | 9.42 | 7.13 | 6.08 | 4.44 | 6.89 |

three has been selected as a suitable number for this work, the results have been calculated in accordance with this figure.

*b. The error of overcrowding.* We now come to consider the second factor determining the optimum number of bacilli to be inoculated in putting up viable counts by the tube method, namely the error of overcrowding. More or less in proportion as the error of sampling decreases as the number of developing colonies increases, so the error of overcrowding increases as the number of developing colonies increases. The two vary in opposite directions; the greater the number of colonies the less the sampling error; the fewer the number of colonies, the less is the overcrowding error. A point must be chosen between the two which will permit of the minimum combined error being experienced. Before this could be done however, it was necessary to ascertain the actual effect of overcrowding on the development of colonies in tube preparations. As mentioned above, this overcrowding error is one which seems to have been neglected by the majority of

observers, or at any rate, not clearly recognized. It is obvious that the greater the number of bacilli distributed in a given space, the less is the interval between each of them, and the greater the chance of two being coincident. In every case in which two bacilli are coincident or are placed very close together only one colony will develop. Further, when two bacilli are situated at such a distance from each other that each is able to develop, yet at such a distance that continued development of both will result in fusion, it is clear that a single colony must arise. Whether one continues to grow and the other desists or whether both develop, the result must be the same—namely, the appearance of one colony in place of two bacilli. On pure à priori grounds one would expect this overcrowding factor to be of considerable importance in determining the number of colonies which will develop in a given space. One would expect it to play but a small part so long as comparatively few bacilli were inoculated, but as the number of the latter increased so should the percentage which fails to develop into colonies become greater. So much for theoretical considerations. It was decided to make a quantitative estimation of this overcrowding factor and the following experiments were performed. A dilution in tap water of a 3 hours' broth culture of *Bact. suipestifer* was put up, of such strength that there was approximately one bacillus in every drop. A series of agar tubes was then inoculated with varying numbers of drops, the tubes being put up in such an order as to equalize the possibly deleterious action of the diluent. As a rule in each experiment, 16 to 20 tubes were inoculated with one drop, 6 to 10 with 5 drops, 6 with 10 drops, 4 with 15 drops, and 4 with 20 drops. After three days' incubation the tubes were counted, and the actual number of colonies developing in the different sets of tubes compared. It is clear that if one colony developed per tube when 1 drop was inoculated, then 5 should develop in the 5-drop tubes, 10 in the 10-drop tubes, 15 in the 15-drop tubes and 20 in the 20-drop tubes. After a number of experiments had been performed with emulsions containing from 1-15 bacilli per drop, another series was instituted in which the emulsions contained from 15 to 30 bacilli per drop and later on, a further series

in which the emulsions contained as many as 30 to 170 per drop. In all 26 experiments were performed. In each experiment the number of colonies which developed in the 5-, 10-, 15-, and 20-drop tubes was calculated as a percentage of the number which developed in the 1-drop tubes, after they had been rendered comparable with these by dividing by the number of drops they respectively contained. An example will make this clear; the number of colonies given represents the arithmetic mean of the counts of the number of tubes used:

TABLE 5

| | NUMBER OF COLONIES IN TUBES CONTAINING | | | | |
|---|---|---|---|---|---|
| | 1 drop | 5 drops | 10 drops | 15 drops | 20 drops |
| Actual count | 77.0 | 371.0 | 690.8 | 918.8 | 1173.8 |
| Count divided by number of drops per tube | 77.0 | 74.2 | 69.08 | 61.25 | 58.69 |
| Percentage of 1-drop counts | 100 | 96.36 | 89.71 | 79.56 | 76.23 |

Here it is seen that the greater the number of organisms inoculated per tube the lower is the actual percentage which develops. In the 1-drop tubes, 77 colonies develop; theoretically therefore, in the 20 drop tubes 1540 colonies should have appeared, but in point of fact only 1173.8 were observed; that is, only 76.23 per cent of the bacilli inoculated formed colonies. The results of each experiment were plotted as a curve, the number of colonies per tube being arranged along the abscissae, and the percentage which developed along the ordinates. In the later curves, starting, for instance, with 100 colonies in the 1-drop tubes, the base could not be taken as 100 per cent, but had to be brought into relation with the results of the earlier curves; reference to these showed that in a tube containing 100 colonies only 97.6 per cent of the bacilli inoculated had developed, and therefore this figure was taken as the starting point for the curve, the other figures in the curve being taken as percentages of this number. Of course, in the earlier curves, when only a few bacilli were being dealt with per tube, the results were very irregular owing to the large sampling error involved; it was hoped that by taking the arithmetic mean of the counts in 16 to 20 tubes, this would be obviated, but

such was not altogether the case. But as the irregularity affected the curves more or less equally in the opposite directions, it is believed that the results obtained by combining all the curves into one composite curve represent the actual facts with a certain degree of accuracy (fig. 2). An examination of this composite curve constructed from the 26 simple curves involved shows that the overcrowding error does not come into play till about 50 bacilli are inoculated per tube. After this point the curve decreases gradually, proceeding obliquely downwards in almost a straight line. Had it been continued far enough, no doubt the obliquity of the descent would have decreased, otherwise at a certain point it would have reached zero, which would manifestly have been absurd. More than 1200 colonies were not counted, as above this point the error due to counting becomes so considerable as to invalidate the results obtained. Indeed any number of colonies greater than 800 per tube renders counting a distinctly arduous procedure, and the curve was continued beyond this point more for theoretical than for practical reasons.

We may now return to the optimum number of colonies desired per tube. It was seen that the sampling and the overcrowding errors varied in opposite directions, and it was therefore necessary to obtain a number which should permit of the minimum error of both. But now that the overcrowding error has been worked out in the form of a curve, it is possible to correct one's results in accordance with this curve, so that this error may be left out of account when the optimum number of colonies per tube is considered. The only factors influencing this point now are the sampling error and the actual error of counting. It was shown before that if only three tubes are used for each count, in order that the deviation of each tube, from the arithmetic mean may not exceed 5 per cent, between 400 and 800 colonies per tube should be aimed at. The counting error is naturally smallest when the colonies are fewest, but it is of little import till the number per tube rises to about 400. This error has been ascertained by counting the same tube twice in succession. The error involved is remarkably small, and not until something like 800 to 1000 colonies are counted does it become appreciable. The time,

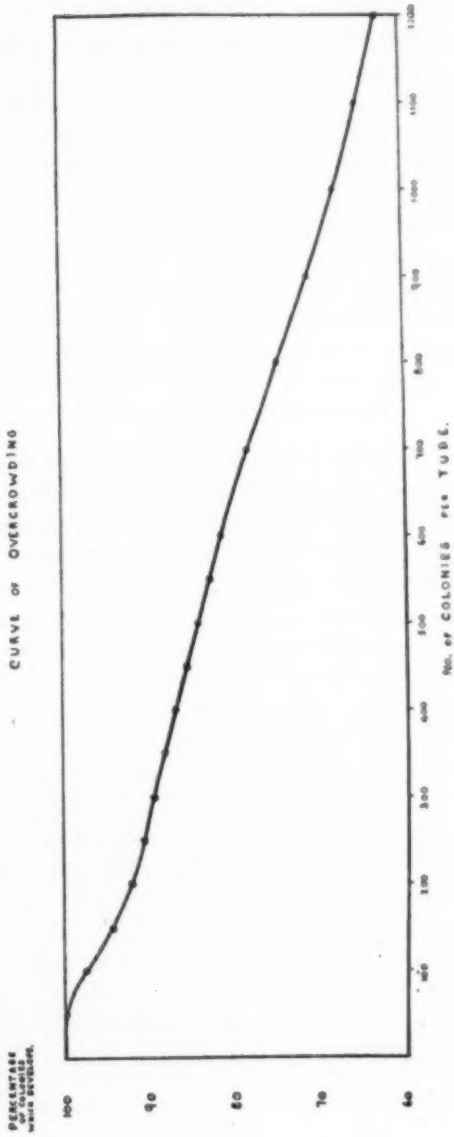
CURVE OF OVERCROWDING
No. of COLONIES PER TUBE.
PERCENTAGE OF COLONIES WHICH DEVELOPED.
100
90
80
70
60

FIG. 2

however, required to count tubes containing this number of colonies is considerable, if several tubes have to be dealt with, and the resulting gain is comparatively small. Taking all factors into consideration then, it would seem that if considerable accuracy is required the optimum number of colonies to be desired per tube is in the neighborhood of 200 to 400, when only three tubes are put up; if a greater number are prepared, the number of bacilli inoculated may be decreased to 100. This will be seen to be somewhat higher than the number advocated by Park and Williams, but taking the experimental data given above as a guide, one feels justifed in regarding this recommendation as more in accordance with the laws of scientific accuracy.

*Technique of the viable count*

Having dealt with these preliminary points, the final technique of the viable count may now be described. Presuming a broth culture of an organism is to be counted, a certain amount of fluid is withdrawn by means of a dropping pipette, and 10 drops are delivered at intervals of one second between each drop into a flask containing a known quantity of sterile tap water at 18°C. After shaking thoroughly, a fresh pipette is used to transfer 10 drops of this emulsion into a second flask, likewise containing a known quantity of tap water at 18°C. The quantity of water in each flask will vary according to the age of the culture which is to be counted; in some cases only 5 cc. will be required, in others as much as 50 or 100 cc.; in any case, the quantity is delivered by means of a carefully calibrated volumetric pipette. When the second flask has been shaken 4, 8 or 12 drops of this emulsion are delivered into three test tubes, measuring 6 inches by $\frac{5}{8}$ inch, each containing about 2 cc. of melted agar at 45°C. The contents are then mixed by gentle shaking, and the tubes are rolled by rotation between the fingers. The tubes are incubated in an inverted position for three days at 37°C., and at the end of that time are counted in the way previously described.

*The error of comparable dilutions*

In order to form an estimate of the actual error of the complete process, 8 experiments were performed in each of which two separate counts of the same emulsion were made under exactly comparable conditions. In each count three tubes were put up and the arithmetic means of these were compared, and the percentage deviation of each set from the arithmetic mean of the two was calculated. The results are shown in table 6.

The results are sufficient to show that the error involved in the viable count performed by the method described is probably

TABLE 6

| EXPERIMENT NUMBER | COUNT OF FIRST SET | COUNT OF SECOND SET | ARITHMETIC MEAN OF TWO SETS | PERCENTAGE DEVIATION |
|---|---|---|---|---|
| | | | | *per cent* |
| 1 | 229,000,000 | 203,500,000 | 216,300,000 | 5.92 |
| 2 | 228,400,000 | 229,800,000 | 229,100,000 | 0.306 |
| 3 | 339,500,000 | 393,100,000 | 366,300,000 | 7.31 |
| 4 | 198,200,000 | 196,200,000 | 197,200,000 | 0.50 |
| 5 | 494,500,000 | 483,900,000 | 489,200,000 | 1.08 |
| 6 | 187,800,000 | 188,000,000 | 187,900,000 | 0.053 |
| 7 | 342,100,000 | 302,000,000 | 322,100,000 | 6.24 |
| 8 | 423,500,000 | 427,700,000 | 425,600,000 | 0.049 |
| Mean percentage deviation | | | | 2.68 |

somewhere in the neighborhood of 5 per cent. For practical purposes this seemed to be sufficiently small to render the method applicable to the study for which it was designed.

Before passing on, however, it is interesting to note that the actual counts obtained of the number of living organisms in a broth culture of *Bact. suipestifer* have been found to be considerably higher than those of many previous observers. Penfold, for instance, working with *Bact. coli* in peptone water, records a maximum of not more than 9,500,000 organisms per cc., while by the method employed in this work, counts of over 700,000,000 organisms per cc., have been met with. Whether such a difference can be ascribed merely to the nature of the medium and to the method employed in counting must be left an open point.

PART II

*Comparison of the total and viable counts*

The primary object of this work was to make a comparison of the total and viable counts, especially during the logarithmic phase of growth, in order to ascertain if they were equal. From a survey of previous work on the subject, one was led to expect that all the bacilli would be found to be alive during this period. To test this a series of 16 experiments was made. At the start

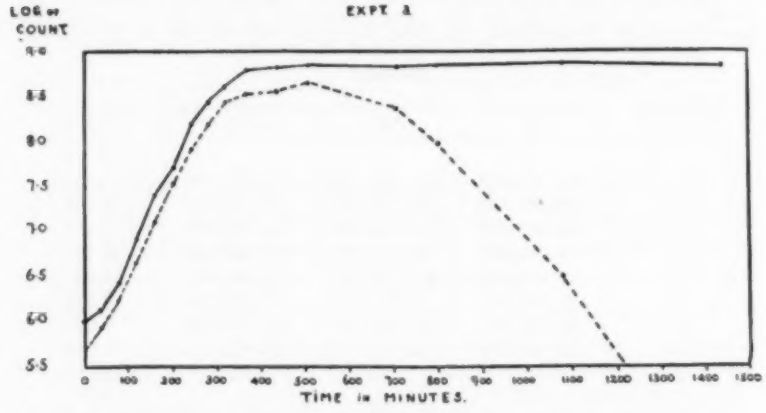


FIG. 3. SHOWING THE TOTAL AND VIABLE COUNTS IN A BROTH CULTURE OF BACT. SUIPESTIFER

Total count = continuous line. Viable count = interrupted line

comparative counts were conducted for the first 24 hours of growth so as to obtain an idea of the general relations of the two curves, but later on the counts were not continued beyond six to nine hours, as by that time the logarithmic phase was already past, and no object in following further progress was to be achieved.

To take one of the first experiments. The technique was as follows: At 6 p.m., in the evening a tube of 5 cc. of broth was inoculated with one loop of a culture of *Bact. suipestifer*, and incubated at 23°C. overnight. At 9.20 a.m. the following morning, after being warmed to 37°C., 10 drops of this culture were used

to seed a flask of 50 cc. of broth also warmed to 37°C. A total and viable count was made on the old culture, and from this the acutal number of bacilli, alive and dead, in the inoculum was calculated. Unfortunately it was only found possible to make one determination of the total count; had three been made and the arithmetic mean taken a closer approximation to the real count would have been obtained. After thorough shaking, the

TABLE 7

*Experiment 8*

| TIME AFTER INOCULATION | VIABLE COUNT PER CUBIC CENTIMETER | TOTAL COUNT PER CUBIC CENTIMETER | RELATION OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | | 962,300 | |
| 40 | 828,800 | 1,286,000 | 64.46 |
| 80 | 1,539,000 | 2,571,000 | 59.84 |
| 120 | 4,363,000 | 8,123,000 | 53.71 |
| 160 | 12,150,000 | 23,970,000 | 50.70 |
| 200 | 32,490,000 | 49,650,000 | 65.45 |
| 240 | 81,640,000 | 155,300,000 | 52.54 |
| 280 | 155,900,000 | 280,000,000 | 55.67 |
| 320 | 271,700,000 | 401,400,000 | 67.70 |
| 370 | 334,300,000 | 606,000,000 | 55.16 |
| 440 | 351,400,000 | 669,500,000 | 52.47 |
| 510 | 454,000,000 | 693,300,000 | 65.49 |
| 710 | 223,700,000 | 647,900,000 | 34.52 |
| 800 | 94,950,000 | 709,600,000 | 13.39 |
| 1080 | 3,134,000 | 702,300,000 | 0.45 |
| 1440 | 7,709 | 658,800,000 | 0.001 |

flask was incubated at 37°C., and total and viable counts were made on it at intervals during the ensuing twenty-four hours. The early total counts were made by the Thoma-Zeiss slide, using methylene blue for staining the organisms, and examining the preparations under open illumination; the later counts were made in the usual manner with the Helbe under dark ground illumination. The results were as shown in table 7 and figure 3.

The irregularity of the total count during the first three hours will be noted. This was due to the unsatisfactory method employed. Until the count reaches about fifty million per cubic

centimeter, when the Helbe chamber can be used, there appears to be no method of insuring accuracy in the results. As variations of 50 per cent and more were encountered by the Thoma-Zeiss method it was decided to abandon this altogether and wait till the numbers were sufficiently great to allow the Helbe to be used. In future experiments, then, no record of the total counts

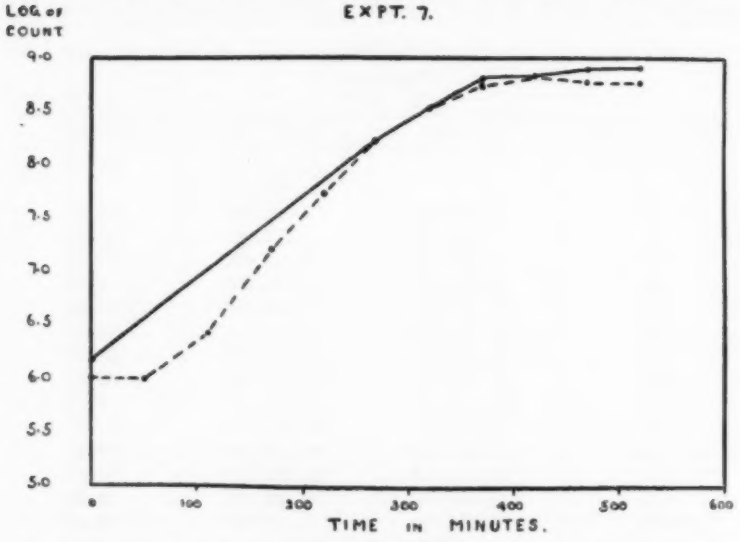


FIG. 4 SHOWING THE TOTAL AND VIABLE COUNTS IN A BROTH CULTURE OF BACT. SUIPESTIFER

Total count = continuous line. Viable count = interrupted line

during the early phases was made. There is no doubt that this is a grave disadvantage, for it prevents one from obtaining figures by which to compare the total and viable counts in the early stages of the logarithmic phase. In practice it was only found possible to obtain one to three comparative counts before the close of the logarithmic phase. As, however, the organisms are dividing more or less uniformly during this phase, it is fair to assume that the relation between the two during the whole of the phase is fairly closely represented by that obtaining during the

latter part. Referring once more to the graph it will be seen that during no part of the growth does the viable count equal that of total. The relation of the two worked out as percentages of the viable to the total is given in the third column of the protocol.

Actually at the end of the logarithmic phase, at two hundred minutes, the relation will be seen to be one of 65.45 per cent. As it has previously been shown that the probable error of either of the counts is not more than about 5 per cent, any question of technical defects falls to the ground.

TABLE 8

*Experiment 7*

| TIME AFTER INOCULATION | VIABLE COUNT PER CUBIC CENTIMETER | TOTAL COUNT PER CUBIC CENTIMETER | RELATION OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 1,024,000 | 1,486,000 | 68.95 |
| 50 | 979,700 | | |
| 110 | 2,631,000 | | |
| 170 | 16,590,000 | | |
| 220 | 54,730,000 | | |
| 270 | 169,100,000 | 162,100,000 | 104.3 |
| 320 | 335,500,000 | 336,700,000 | 99.63 |
| 370 | 581,500,000 | 669,500,000 | 86.84 |
| 420 | 693,300,000 | 691,500,000 | 100.2 |
| 470 | 626,600,000 | 813,000,000 | 77.07 |
| 520 | 603,400,000 | 825,600,000 | 73.08 |

To confirm this, other experiments were performed, the technique being kept constant except for the nature of the inoculum. This was varied in several ways to see whether the previous history of the culture from which the 5 cc. tube of broth was seeded, whether old, recent, or very young, had any effect on the subsequent growth. This was found not to be the case. Again, it was thought that possibly the discrepancy in the relation of the two might be due to the presence of a lag phase, but obliteration of this factor by inoculating the flask of broth from a young culture growing at 37°C. failed to substantiate this either. In other experiments the inoculum was from the last of a series of rapid subcultures of the organism, no subculture being allowed

to grow for more than three hours at 37°C., so as to maintain the organisms in a medium of comparatively fresh broth. This again was without effect. The viable count still persisted below the total. No method, in fact, could be ascertained which would abolish this inferiority, and it was thought that possibly the tech-

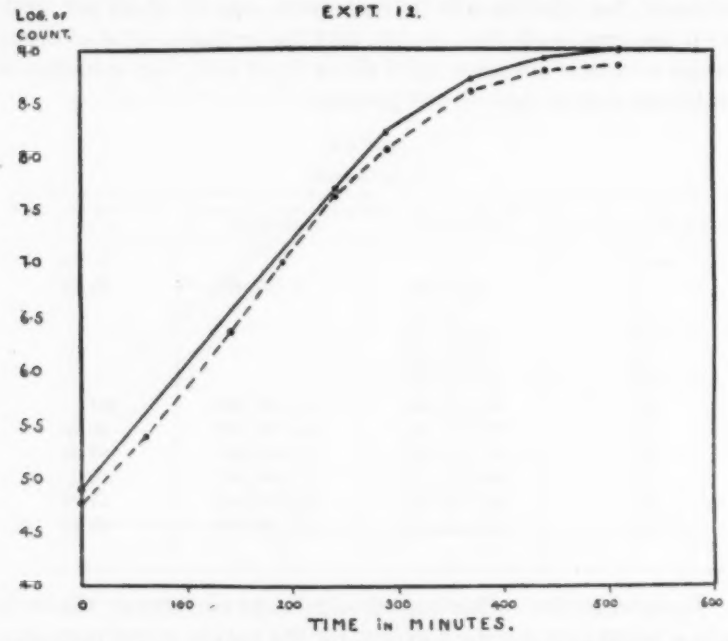


FIG. 5 SHOWING THE TOTAL AND VIABLE COUNTS IN A BROTH CULTURE OF BACT. SUIPESTIFER

Total count = continuous line. Viable count = interrupted line

nique or the calibration of the instruments employed might be at fault. But this idea had likewise to be given up when quite unexpectedly the result of one experiment showed that during at least fifty minutes of growth the two counts were identical. The figures for this experiment are quoted in table 8 and figure 4.

The low total count at four hundred and twenty minutes was probably due to a technical error. Apart from this it will be seen

that the relation of the viable to the total count rises from 68.95 per cent at the start to equality; after remaining at this level for at least fifty minutes, it declines gradually to 73.08 per cent at 520 minutes. The reason for this equality was not forthcoming, but that it, too, was not due to a technical defect was shown by the fact that in three other experiments a similar equality of the total and viable counts was met with. No amount of analysis of these curves has yet succeeded in demonstrating the reason for the difference in the results between these four and the other twelve experiments. The previous history of the culture, the

TABLE 9

*Experiment 12*

| TIME AFTER INOCULATION | VIABLE COUNT PER CUBIC CENTIMETER | TOTAL COUNT PER CUBIC CENTIMETER | RELATION OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 62,150 | 81,470 | 76.28 |
| 60 | 249,700 | | |
| 140 | 2,417,000 | | |
| 190 | 10,570,000 | | |
| 240 | 43,290,000 | 50,780,000 | 85.23 |
| 290 | 116,900,000 | 176,000,000 | 66.37 |
| 370 | 416,100,000 | 535,100,000 | 77.76 |
| 440 | 646,800,000 | 860,400,000 | 75.16 |
| 510 | 751,500,000 | 1,045,000,000 | 71.90 |

size of the inoculum, the relation between the total and viable counts in the inoculum, the presence or absence of lag, the duration of the logarithmic phase, the actual increase in numbers during the logarithmic phase, and the rate of growth have all been studied without showing any definite correlation between these factors in the 4 experiments quoted.

In conclusion one more example will be given in which at the end of the logarithmic phase the relation between the total and viable counts was as high as 85.23 per cent (table 9 and fig. 5).

Here it will be seen that the condition is intermediate between the two curves already shown. Unfortunately from lack of space it is impossible to reproduce the curves for each of the sixteen counts, but the individual protocols will be found in tables 10 to 22.

TABLE 10

*Experiment 1*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 1,586,000 | 961,000 | 60.57 |
| 60 | | 1,199,000 | |
| 120 | | 6,337,000 | |
| 180 | 50,900,000 | 39,680,000 | 77.94 |
| 240 | 141,900,000 | 118,400,000 | 83.43 |
| 300 | 723,600,000 | 381,500,000 | 52.71 |
| 360 | 967,900,000 | 617,500,000 | 63.80 |

TABLE 11

*Experiment 2*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 1,327,000 | | |
| 50 | | 1,350,000 | |
| 90 | | 3,522,000 | |
| 130 | | 10,320,000 | |
| 165 | 53,190,000 | 24,200,000 | 45.50 |
| 200 | 130,100,000 | 45,500,000 | 34.97 |
| 240 | 234,200,000 | | |
| 295 | 360,300,000 | 241,600,000 | 67.05 |
| 360 | 628,700,000 | 509,300,000 | 81.00 |

TABLE 12

*Experiment 4*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 108,400 | | |
| 50 | | 181,200 | |
| 100 | | 446,300 | |
| 150 | | 1,345,000 | |
| 220 | | 8,846,000 | |
| 260 | 51,350,000 | 29,710,000 | 57.86 |
| 300 | 107,600,000 | 69,490,000 | 64.52 |
| 340 | 206,500,000 | 104,600,000 | 50.63 |
| 375 | 271,100,000 | 163,300,000 | 60.24 |

TABLE 13

*Experiment 5*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 584,100 | | |
| 40 | | 972,900 | |
| 90 | | 2,129,000 | |
| 132 | | 4,506,000 | |
| 175 | | 10,560,000 | |
| 220 | | 34,430,000 | |
| 270 | 93,930,000 | 86,540,000 | 92.12 |
| 310 | 189,500,000 | 148,600,000 | 78.38 |
| 360 | 361,200,000 | 266,700,000 | 73.82 |
| 405 | 496,300,000 | 371,000,000 | 74.76 |

TABLE 14

*Experiment 6*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 959,600 | 913,500 | 95.19 |
| 50 | | 1,495,000 | |
| 100 | | 2,530,000 | |
| 150 | | 7,954,000 | |
| 200 | | 19,780,000 | |
| 250 | | 68,100,000 | |
| 300 | (?) 119,300,000 | 146,700,000 | (?) 122.80 |
| 350 | 227,700,000 | 229,900,000 | 100.90 |
| 390 | 417,000,000 | 328,300,000 | 78.74 |
| 450 | 627,900,000 | 464,300,000 | 73.94 |

TABLE 15

*Experiment 8*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 1,851,000 | 1,692,000 | 91.41 |
| 50 | | 1,852,000 | |
| 110 | | 2,101,000 | |
| 170 | | 6,548,000 | |
| 220 | | 24,740,000 | |
| 270 | 76,630,000 | 49,140,000 | 64.12 |
| 320 | (?) 252,400,000 | 102,400,000 | (?) 40.58 |
| 370 | 365,100,000 | 230,700,000 | 63.19 |
| 420 | 526,200,000 | 361,400,000 | 68.68 |
| 470 | 771,300,000 | 584,500,000 | 75.78 |

TABLE 16

*Experiment 9*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 726,300 | 501,800 | 69.08 |
| 50 | | 892,300 | |
| 110 | | 2,460,000 | |
| 170 | | 7,802,000 | |
| 220 | | 18,410,000 | |
| 270 | 75,900,000 | 59,570,000 | 78.48 |
| 320 | 167,100,000 | 131,500,000 | 78.66 |
| 370 | 361,200,000 | 235,100,000 | 65.09 |
| 420 | 564,400,000 | 423,100,000 | 74.96 |
| 470 | 653,300,000 | 490,500,000 | 75.08 |

TABLE 17

*Experiment 10*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 1,020,000 | 869,800 | 85.33 |
| 50 | | 1,409,000 | |
| 110 | | 2,944,000 | |
| 170 | | 11,700,000 | |
| 220 | | 26,650,000 | |
| 270 | 89,780,000 | 66,160,000 | 73.69 |
| 320 | 238,900,000 | 135,400,000 | 56.67 |
| 370 | 388,600,000 | 287,200,000 | 73.91 |
| 420 | 533,400,000 | 430,300,000 | 80.67 |
| 470 | 854,700,000 | 558,600,000 | 65.36 |

TABLE 18

*Experiment 11*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 1,303,000 | 1,198,000 | 91.89 |
| 50 | | 1,699,000 | |
| 110 | | 2,983,000 | |
| 170 | | 12,480,000 | |
| 220 | 53,440,000 | 35,690,000 | 66.77 |
| 270 | 156,000,000 | 111,000,000 | 71.15 |
| 320 | 345,500,000 | 238,700,000 | 69.08 |
| 370 | 563,700,000 | 339,900,000 | 60.30 |
| 420 | 731,300,000 | 451,200,000 | 61.70 |
| 470 | 840,300,000 | 553,400,000 | 65.86 |

TABLE 19

*Experiment 13*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 272,000 | 201,500 | 74.08 |
| 60 | | 561,600 | |
| 140 | | 5,141,000 | |
| 190 | | 24,790,000 | |
| 240 | | 121,100,000 | |
| 290 | 269,300,000 | 280,300,000 | 104.10 |
| 350 | 487,400,000 | 484,400,000 | 99.38 |
| 400 | 834,900,000 | 598,900,000 | 71.74 |

TABLE 20

*Experiment 14*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 120,300 | 122,600 | 101.80 |
| 60 | | 357,300 | |
| 140 | | 2,094,000 | |
| 190 | | 10,020,000 | |
| 230 | 46,260,000 | 29,270,000 | 63.27 |
| 250 | 64,600,000 | 50,340,000 | 77.94 |
| 270 | 89,190,000 | 67,670,000 | 75.88 |
| 350 | 364,100,000 | 273,200,000 | 75.02 |

TABLE 21

*Experiment 15*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 132,000 | 85,920 | 64.94 |
| 130 | | 3,045,000 | |
| 210 | 47,950,000 | 42,010,000 | 87.60 |
| 230 | 64,030,000 | 56,930,000 | 88.90 |
| 250 | 93,930,000 | 82,300,000 | 87.62 |
| 290 | 217,600,000 | 160,800,000 | 73.87 |
| 380 | 487,400,000 | 439,300,000 | 90.14 |

TABLE 22

*Experiment 16*

| TIME | TOTAL COUNT PER CUBIC CENTIMETER | VIABLE COUNT PER CUBIC CENTIMETER | PERCENTAGE OF VIABLE TO TOTAL |
|---|---|---|---|
| *minutes* | | | *per cent* |
| 0 | 69,130 | 68,930 | 99.70 |
| 40 | | 170,500 | |
| 160 | | 5,585,000 | |
| 210 | | 22,360,000 | |
| 225 | 55,440,000 | 39,090,000 | 70.52 |
| 240 | (?) 76,740,000 | 69,950,000 | (?) 91.16 |
| 260 | 111,100,000 | 81,850,000 | 73.69 |
| 280 | 196,700,000 | 116,500,000 | 59.23 |
| 390 | 522,600,000 | 417,100,000 | 79.80 |

## DISCUSSION

How, it may be asked, can the discrepancy between the total and viable counts during the logarithmic phase of growth be explained? It is only necessary to consider three hypotheses. (1) It might be referred to technical errors; that this is not so has been shown already. Moreover the uniformity of the individual counts suffices to alleviate one's few remaining suspicions on this point. (2) It might possibly be concerned with a method of reproduction other than that by binary fission. If, for instance, there were during certain phases of the culture a multiplication of the organisms by a process of budding or even by a sexual mode of some kind, it is conceivable that a certain number of parent cells might die or pass into a resting stage, and thus lead to the presence of a proportion of dead or inactive bacteria in the medium, even during the height of growth. For this, however, there is no direct evidence. Division of the particular strain of *Bact. suipestifer* used has been watched for several generations in gelatin film preparations on a large number of occasions, and never has any other method of reproduction than that by binary fission been encountered. (3) The most likely explanation would appear to be that of every generation produced a certain number of bacteria die. If, for example, 80 per cent of the organisms produced during a given generation continued

to live and divide, while 20 per cent failed to do so, the result would be that at the end of the logarithmic phase the total number of organisms alive and dead would exceed the number of living organisms. Further, the increase in the living organisms would still occur by geometrical progression, and the resultant curve of plotting the logarithms of the numbers would still fall on a straight line; the only difference would be that instead of the number of organisms being doubled in each generation, they would only be increased 1.6 times as much. To make this clear, suppose there are 1000 organisms per cubic centimeter, alive at the commencement of the logarithmic phase. At the end of the first generation there would be 2000 organisms, of which 80 per cent or 1600 would live and divide, while 20 per cent or 400 would

TABLE 23

| | NUMBER OF VIABLE ORGANISMS PER CUBIC CENTIMETER | NUMBER OF DEAD ORGANISMS PER CUBIC CENTIMETER | TOTAL NUMBER OF ORGANISMS PER CUBIC CENTIMETER |
|---|---|---|---|
| At start | 1,000 | 0 | 1,000 |
| At end of first generation | 1,600 | 400 | 2,000 |
| At end of second generation | 2,560 | 640 | 3,200 |
| At end of third generation | 4,096 | 1,024 | 5,120 |

die. In the next generation these 1600 would divide and produce 3200 organisms of which again 20 per cent or 640 would die, while 2560 would live and divide and so on. In tabular form it may be seen more clearly still.

It will be noticed that the living organisms increase in each generation by 1.6 times as many as those present at the commencement. On the other hand the factors of increase for both dead and total organisms start high and gradually decrease till after several generations they approximate to 1.6. If curves were plotted of the logarithms of these counts, it would be seen that the curve for the viable organisms would lie along an ascending oblique straight line, whereas that for the total would rise at first rapidly and then gradually become flatter till it ran almost parallel to that for the viable count. Whether this is so in practice cannot be ascertained, as no practicable method of counting total numbers of organisms during the early part of the

logarithmic phase has been devised. So far, however, as the latter part of the phase is considered, it is seen that the total and viable curves do actually run very nearly parallel to one another. Whether or no this hypothesis be correct it does at least seem to explain how a dissociation of the total and viable counts may occur, and how the logarithmic curve for the viable count can yet ascend by means of a straight line—a fact which has been substantiated by several workers independently. Of course the actual percentage of the viable to the total is not constant, the figures in the 16 experiments undertaken varying from 57.86 to 122.8 per cent. Taking the arithmetic mean of the percentage relations between the two counts at the end of the logarithmic phase, the proportion in the 16 experiments came to 81.42 per cent. It seems justifiable to conclude therefore that under the ordinary conditions of in vitro cultures of *Bact. suipestifer* there is a normal death rate of approximately 20 per cent per generation, with variations extending between 43 per cent on the one hand to nil on the other. If this is actually so, it is a very surprising fact—totally in disaccord with all previous work on the subject. Translated into actual figures it means that no fewer than 200 organisms in every 1000 produced are dying in each generation. The old teaching has been accepted for so long—namely, that during the logarithmic phase all the bacilli are viable that one naturally feels diffident in contradicting it; yet the figures quoted seem to admit of no other course. Moreover, viewed from a general biological standpoint, it would be surprising if all the progeny in a given species continued to live and reproduce, however favorable the environment and however abundant the food supply. It seems to be very much more in accordance with the principles of this science that a certain proportion of each generation should fail to attain maturity and to propagate their kind; what the actual cause of this may be is difficult to ascertain, but the fact remains that in practically every species studied there is a variation in the progeny leading to the survival of some and the death of the others.

*Calculation of the generation time*

Taking for granted that there is a normal death rate of the organisms in vitro cultures, it is immediately apparent that the generation time must be calculated afresh. Hitherto the number of generations occurring during a given period has been calculated from the formula $n = \frac{\log b - \log a}{\log 2}$ where $n$ = the number of generations, $b$ = the number of organisms at the end of the given period, and $a$ = the number alive at the beginning of that period (Chesney, 1916). This formula has been employed solely on the assumption that the number of organisms doubles in each generation. But if, for instance, only 80 per cent are dividing, then the increase in the number of organisms in each generation will only rise by 1.6. Hence the formula must be altered to $n = \frac{\log b - \log a}{\log 1.6}$ The result of this will be to give a larger number of generations in the particular period studied, and consequently a shorter time for the production of each generation. It is now seen that the generation time cannot be calculated with accuracy unless the number of both the total and viable organisms be known. In these experiments these numbers are known, at least for the latter part of the logarithmic phase. What has been done then, is to estimate the relation between the total and viable organisms at the end of the logarithmic phase. Assuming that this relation has persisted all through the phase—and it is difficult to dispute the probability of this considering that the viable count increases regularly—one can then deduce the percentage of organisms which has remained alive and divided in each generation, and hence the total number of generations. For instance, reverting to the figures given for experiment 12 (table 9). The number of organisms alive at the commencement of the logarithmic phase, namely, 0 minutes, was 62,150 per cubic centimeter. At 240 minutes, the close of the logarithmic phase, there were 43,290,000 per cubic centimeter living and 50,780,000 per cubic centimeter total alive and dead. The proportion of viable to total is then 85.23 per cent. The actual increase in the viable count per

generation must therefore have been 1.71. Using the formula of $n = \frac{\log b - \log a}{\log 1.71}$ it is found that in 240 minutes there were 12.2 generations, which gives a generation time of 19.7 minutes. Calculated by the old formula $n = \frac{\log b - \log a}{\log 2}$ the number of generations is only 9.4, and the generation time is 25.5 minutes. It is clear therefore that the generation time is in general shorter that that hitherto recorded. To give an idea of the probable figure, the average generation times for the whole 16 counts during the logarithmic phases has been worked out; it comes to 21.05 minutes.

Taking all the facts into consideration, it seems that in cultures of *Bact. suipestifer* there is a normal death rate, even during the period when the maximum rate of growth is proceeding. The extent of this will vary in individual cultures. In some it is as high as 43 per cent, in others it is only 20 per cent or 10 per cent, while finally in a few it is for a short period actually nil.

## CONCLUSIONS

1. A method for estimating the number of the total and the viable organisms in a culture medium is described. It is claimed that the experimental error involved in each count probably does not exceed 5 per cent.

2. Applying this to the study of the relation existing between the living organisms in a culture and the total number of organisms alive and dead, it is seen that even during the logarithmic phase the percentage of viable organisms seldom rises above 90 per cent of the total.

3. To explain this a hypothesis is advanced which supposes that in an in vitro culture of *Bact. suipestifer* there is a normal death rate amongst the bacteria, even during the logarithmic phase of growth.

4. Assuming the presence of a normal death rate, it has been possible to calculate the generation time on an altered basis, and

this time has been found to be shorter than that usually given for cultures of similar organisms.

It is with pleasure that I record my thanks firstly to Dr. W. W. C. Topley for his constant help and advice throughout the progress of this work, and secondly to Mr. J. E. Barnard, of the National Institute of Medical Research, for his assistance in the calibration of the Helbe counting chamber employed in these investigations.

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# A METHOD OF DETECTING RENNET PRODUCTION BY BACTERIA

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The usual method for determining whether bacteria produce rennet or rennet-like enzymes is to inoculate the organisms under investigation into tubes of sterilized milk and then to notice whether or not the milk curdles without the production of acid. This method has several weaknesses. Heated milk is not as readily curdled by enzymes as unheated milk and the curd produced is often so soft that it can scarcely be detected; when an organism produces peptonizing enzymes as well, the presence of curd before peptonization is often difficult to demonstrate; certain organisms curdle the milk through the action of enzymes and subsequently bring about an acid reaction, so that it is difficult to tell whether the milk has been coagulated by the acid or by an enzyme.

It is possible by the following very simple method to avoid these weaknesses of the usual technic. Inoculate the culture under investigation into the milk in the usual manner; then incubate for twenty-four hours or such time as is necessary to allow the organism in question to produce vigorous action in the milk with at least 0.5 cc. of whey on the surface. At the end of this incubation period obtain fresh milk and place 10 cc. of it in a test tube, but do not sterilize it. Warm this milk to about 37°C. Then add to the milk a measured quantity, say 0.5 cc., of whey from the incubated culture and place in a 37° incubator. Examine every five minutes for the first half hour and, if not curdled then, at less frequent intervals for a few hours longer. If rennet is present in any abundance, the milk is ordinarily curdled inside of half an hour. By varying the quantity

of whey added to the milk from 0. 1 cc. up to 1cc. and by recording the time necessary for coagulation, it is possible to make comparisons between different cultures on a quantitative basis.

The advantages of this method are as follows: the unheated milk curdles readily and forms a typical firm rennet curd; the peptonizing enzymes act more slowly and do not in any way obscure the curdling action of the rennet; the period of observation is so short that neither the bacteria present in the milk nor those added in the cultures have time to act on the milk themselves and the amount of whey added is too small to precipitate the casein by the action of any acid it might contain, hence the reaction occurring is due to the action of enzymes alone. By the use of this method it has been found possible to obtain typical rennet curds from certain organisms ordinarily producing both acid and rennet and from others ordinarily digesting the milk so rapidly that no true curd could be observed. By the usual method the production of rennet by these organisms was suspected but could not be actually demonstrated.

This technic is offered as an addition to the methods of pure culture study in use in connection with the society's descriptive chart.